REVERSAL OF CHRONIC PAIN BY ACTIVATION OF SINGLE $GABA_A$ RECEPTOR SUBTYPES

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SUMMARY

The ability to perceive pain is a basic property of humans and of all other higher order animals. The primary role of pain sensation and nociception (that is, the neuronal activity encoding pain) is to protect against potentially harmful threats arriving from the environment or the body interior. Pain can however also become dysfunctional and persist for extended periods of time without an apparent benefit, instead becoming a major burden that requires medical attention. Chronic pain is a major socio-economic challenge, which – despite scientific advances in the understanding of its causes – remains poorly responsive to the drugs available on the market today. Recent insights into the mechanisms of chronic pain states suggest that a common factor for many kinds of persistent pain states is a loss of inhibition in spinal cord circuits that normally control nociceptive input to the brain. The so-called benzodiazepines – first marketed by Hoffmann-La Roche in the 1960s – are drugs that facilitate synaptic inhibition throughout the CNS and have as such the potential to reverse pathological disinhibition. Benzodiazepines facilitate inhibition by increasing the activity of γ-aminobutyric acid (GABA) at its receptor, a heteropentameric anion permeable ion channel. Although rodent studies have shown that pathologically increased pain sensitivity can be normalized by intrathecal (spinal) injection of benzodiazepines, these drugs do not exert clinically relevant analgesia in human patients, at least not after systemic application.

In this thesis, I have tested the hypothesis that benzodiazepines reverse pathological pain after systemic application if their action is restricted to well-defined subtypes of $GABA_A$ receptors. Using $GABA_A$ receptor point-mutated mice, I was able to demonstrate that selective targeting of $GABA_A$ receptors that contain the α2 subunit (α2-$GABA_A$) receptors evoke pronounced pain relief in the absence of confounding and undesired sedation. I could also confirm previous findings that had proposed that activation of the same $GABA_A$ receptors induces anxiolysis and muscle relaxation. Importantly, selective targeting of α2-$GABA_A$ receptors avoided several unwanted effects of classical non-selective benzodiazepines including sedation, impairment of motor coordination, and the progressive loss of therapeutic efficacy over time. Using mice in which the action of classical benzodiazepine agonists was restricted to only a single $GABA_A$ receptor subtype, I could also propose a new hypothesis explaining why classical benzodiazepines lack clinically relevant analgesic properties. For two clinically used benzodiazepines (diazepam and midazolam), I could demonstrate that strong α1-$GABA_A$ receptor-mediated sedation occurs already at doses

more than 20 times lower that those required for significant analgesia. Dose limiting sedation is therefore the likely reason for the lack of clinically relevant analgesia in human pain patients.

The second part of the thesis project has been designed to foster the translation of the results of the first project to clinical application. Within recent years, drug companies have developed benzodiazepine site agonists with improved subtype specificity (that is, with reduced activity at α1-$GABA_A$ receptors). So far, none of these compounds is generally available for tests in humans, prompting for alternative strategies to obtain proof-of-concept evidence. A previous report has suggested that N-desmethyl clobazam (NDMC), a major metabolite of the clinically used benzodiazepine clobazam, may have a pharmacological profile more favorable than that of most classical benzodiazepines. We found that NDMC indeed possesses an improved α2 versus α1-$GABA_A$ receptor selectivity *in vitro*, and pronounced antihyperalgesic efficacy at doses that do not induce apparent sedation or impairment of motor coordination in mice. In the light of this favorable pharmacological profile and given the fact that new unexpected side effects are highly unlikely to occur in a metabolite of a clinically used benzodiazepine, NDMC should be well-suited for proof-of-concept trials in human volunteers or pain patients.

The results of this thesis thus provide further support for a potential use of α2-selective $GABA_A$ receptor modulators as novel analgesics.

ZUSAMMENFASSUNG

Schmerzwahrnehmung ist eine grundlegende Fähigkeit des Menschen und aller höheren Tiere. Die primäre Funktion des Schmerzes und der Nociception (d.h. der neuronalen Kodierung von Schmerz) ist der Schutz vor potentieller Gewebeschädigung durch exogene oder endogene Einflüsse. Schmerzen können sich jedoch auch zu einer Fehlfunktion des Körpers entwickeln und über längere Zeit ohne offensichtlichen Nutzen bestehen bleiben. Schmerzen werden dann zu einer schweren Belastung für das Wohlbefinden der Betroffenen. Obwohl bedeutende Fortschritte in unserem Verständnis der biologischen Ursachen chronischer Schmerzen gemacht wurden, ist ihre Pharmakotherapie immer noch unbefriedigend. Relativ kürzlich gewonnene Einsichten in die zugrunde liegenden Mechanismen legen nahe, dass viele Formen persistierender Schmerzen mit einer Verminderung der synaptischen Hemmung in spinalen Schmerz verarbeitenden Schaltkreisen einhergehen. Benzodiazepine, die erstmals von der Firma Roche in den 1960er Jahren auf den Markt gebracht wurden, sind Medikamente, die die synaptische Hemmung in den meisten Arealen des ZNS verstärken, indem sie die Bindung des hemmenden Neurotransmitters γ-Aminobuttersäure (GABA) an seine Rezeptoren, die $GABA_A$ Rezeptoren, erhöht. Solche Substanzen sollten grundsätzlich in der Lage sein, eine pathologisch verminderte synaptische Hemmung zu korrigieren. Obwohl Studien in Nagern tatsächlich gezeigt haben, dass pathologisch gesteigerte Schmerzempfindlichkeit durch intrathekal injizierte Benzodiazepine normalisiert werden kann, vermitteln diese Wirkstoffe beim Patienten keine klinisch relevante Analgesie.

In dieser Arbeit wurde untersucht, ob Benzodiazepine nach systemischer Applikation gegen pathologische Schmerzen wirksam sind, wenn ihre Wirkung auf bestimmte Subtypen von $GABA_A$ Rezeptoren beschränkt wird. Mit Hilfe von $GABA_A$ Rezeptor-punktmutierten Mäusen konnte gezeigt werden, dass selektive Aktivierung von α2-$GABA_A$ Rezeptoren eine ausgeprägte Analgesie hervorruft, ohne gleichzeitig unerwünschte und die Ergebnisse verfälschende Sedation hervorzurufen. Die neuen Ergebnisse bestätigen zudem, dass die Aktivierung derselben Rezeptoren auch anxiolytisch und muskelrelaxierend wirkt. Die Beschränkung der Wirkung auf α2-$GABA_A$ Rezeptoren erlaubt ausserdem mehrere unerwünschte Wirkungen von klassischen nicht-selektiven Benzodiazepinen zu vermeiden, einschliesslich Sedation, Beeinträchtigung der Motorkoordination, und Toleranzentwicklung

(d.h. den allmählichen Wirkungsverlust während chronischer Behandlung). Experimente mit diesen und weiteren Mäusen, bei denen die Wirkung von klassischen Benzodiazepinen auf einen einzigen $GABA_A$ Rezeptorsubtyp beschränkt war, konnten zudem eine Erklärung dafür liefern, warum mit klassischen Benzodiazepinen keine klinisch relevante Analgesie erzielt werden kann. Am Beispiel der beiden klassischen Benzodiazepine Diazepam und Midazolam konnte gezeigt werden, dass α1-$GABA_A$ Rezeptor-vermittelte Sedation bereits in Dosierungen auftritt, die 20-fach unter denen liegen, die für eine signifikante Analgesie benötigt werden. Die durch klassische Benzodiazepine hervorgerufene Sedation verhindert somit, dass analgetisch wirksame Dosen unter therapeutischen Bedingungen verabreicht werden können.

Der zweite Teil dieses Projekts widmete sich der Translation der Ergebnisse des ersten Projekts in Richtung auf eine klinische Anwendung. Pharmazeutische Firmen haben in den letzten Jahren einige Benzodiazepinrezeptoragonisten mit verbesserter Subtyp-Selektivität (d.h. mit verminderter Aktivität an α1-$GABA_A$ Rezeptoren) entwickelt. Bisher ist keine dieser Substanzen für Studien am Menschen allgemein verfügbar, was die Suche nach Alternativen für „proof-of-concept“ Studien veranlasst hat. Eine frühere Arbeit berichtete, dass N-Desmethylclobazam (NDMC), einer der Hauptmetaboliten des klinisch verwendeten Clobazam, ein bessere pharmakologisches Profil haben könnte als die meisten klassischen Benzodiazepine. In der vorliegenden Arbeit konnte gezeigt werden, dass NDMC *in vitro* tatsächlich α2-$GABA_A$ Rezeptoren stärker aktiviert als α1-$GABA_A$ Rezeptoren. In Verhaltenstestes an Wildtypmäusen zeigte NDMC darüber hinaus eine ausgeprägte analgetische Wirksamkeit in Dosierungen, die keine offensichtliche Sedation oder Beeinträchtigung der Motorkoordination verursachten. Angesichts dieses besseren pharmakologischen Profils und unter Berücksichtigung der Tatsache, dass das Auftreten von neuen, mit Clobazam nicht beobachteten unerwünschten Wirkungen unwahrscheinlich ist, sollte NDMC eine für „proof-of-concept“ Studien am Menschen geeignete Substanz sein.

Zusammenfassend unterstützen die Ergebnisse dieser Dissertation eine mögliche Verwendung von selektiven α2-$GABA_A$ Rezeptormodulatoren als neuartige Analgetika.

ABBREVIATIONS AND CHEMICAL NAMES

5-HT, 5-hydroxytryptamine

AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BDNF, brain-derived neurotrophic factor

BOLD, blood oxygenation level dependent

CCI, chronic constriction injury

CFA, complete Freund's adjuvant

CGRP, calcitonin gene related peptide

COX, cyclooxygenase

CTB, cholera toxin B subunit

DMCM, methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate

DRG, dorsal root ganglion

EP2, prostaglandin E receptor type 2

fMRI, functional magnetic resonance imaging

$GABA_AR$, γ-aminobutyric acid type A receptor

GAD, glutamate decarboxylase

GPCR, G protein coupled receptor

HDAC, histone deacetylase

HZ166, ethyl 8-ethynyl-6-(pyridin-2-yl)-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate

IB4, isolectin B4

IPSC, inhibitory postsynaptic current

KCC2, potassium chloride transporter 2

L-838,417, 7-tert-Butyl-3-(2,5-difluoro-phenyl)-6-(2-methyl-2H-[1,2,4]triazol-3-ylmethoxy)-[1,2,4]triazolo[4,3-b]pyridazine

LTD, long-term depression

LTP, long-term potentiation

mGluR, metabotropic glutamate receptor

MK-0343, 7-cyclobutyl-6-(2-methyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2,6-difluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine

MRK-409, 7-cyclobutyl-6-(2-methyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2,6-difluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine

NDMC, N-desmethyl clobazam

NGF, nerve growth factor

NK1, neurokinin 1

NMDA, N-methyl D aspartate

NS11394, 3'-[5-(1-Hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitrile

NSAID, non-steroidal anti-inflammatory drug

PAG, periaqueductal grey

PG, prostaglandin

PKA, protein kinase A

PSNL, partial spinal nerve ligation

RVM, rostroventromedial medulla

SNI, spared nerve injury

SNL, spinal nerve ligation

SNT, sciatic nerve transection

SP, substance P

SSNRI, selective serotonin and noradrenaline re-uptake inhibitor

TNT, tibial nerve transection

TPA023, 7-(1,1-Dimethylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine

TPA023B, 6,2′-difluoro-5′-[3-(1-hydroxy-1-methylethyl)imidazo[1,2-b][1,2,4]triazin-7-yl]biphenyl-2-carbonitrile

trkA, tyrosine receptor kinase A

TRPA, transient receptor potential ankyrin

TRPM, transient receptor potential M

TRPV, transient receptor potential channel vanilloid type

VGLUT, vesicular glutamate receptor transporter

α_x-GABA$_A$R, GABA$_A$ receptor containing the α_x subunit

GENERAL INTRODUCTION

PAIN

Pain is the conscious experience that occurs in our brain as a consequence of *nociception*. Nociception is the response of the peripheral nervous system to encountered harmful stimuli in our environment. Such stimuli may be noxious heat or cold, bruising mechanical pressures and irritant chemicals. The ability to perceive acute noxious threats increases our chances of survival by adding to our awareness and by triggering protective reflexes that occur unconsciously and almost instantaneously. For instance, immediately withdrawing a hand from a hot plate in the kitchen protects from tissue damage. This is obviously a protective feature preserved and refined throughout evolution, as evidenced by a reduced life span of patients with congenital insensitivity to pain (Cox et al., 2006). Pain can also turn persistent and dysfunctional, causing major debilitation and reduce quality of life.

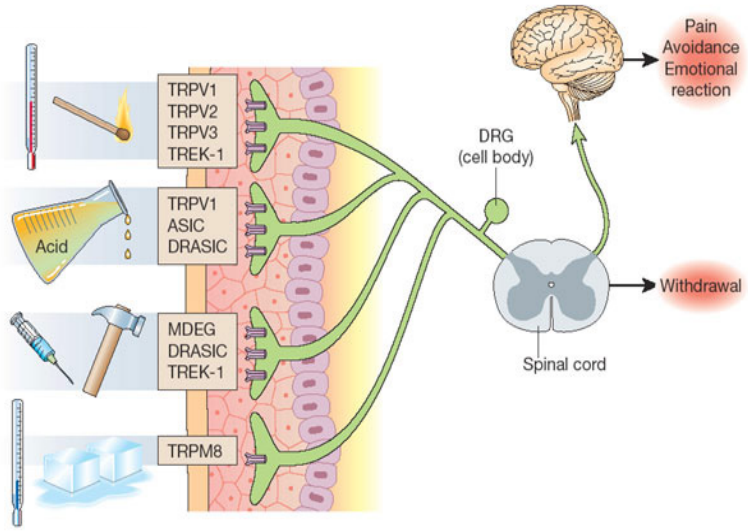

Figure 1. Pain pathway – from skin to spinal cord to brain, adapted from (Scholz and Woolf, 2002).
The thinly myelinated Aδ fibers and unmyelinated C fibers react to various kinds of potentially damaging stimuli in the environment. This can be high or low temperatures, liquids with high concentration of protons or strong mechanical stress or skin-perforating needles. Receptors responding to these stimuli depolarize the peripheral terminal of the primary afferent and send the information along the primary afferent fiber to the spinal cord dorsal horn. From here, the information travels up to the brain where the painful sensation experienced. Connected nuclei in the brain are subsequently activated, triggering emotional, avoidance and other memory-related outcomes.

Pain has been defined in 1979 by John Bonica "as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Bonica, 1979). Any pain-inducing process occurring before the conscious experience of pain is therefore not referred to as pain but nociception. Noxious stimuli such as very cold or hot temperatures, intense mechanical stimuli, irritant chemicals, low pH, are sensed by dedicated sensory neurons, called primary nociceptors (Figure 1).

These convert noxious stimuli into electrical signals (first in generator potentials and subsequently in action potentials) and relay these signals to the spinal cord or brain stem. From there, the information is further relayed to higher CNS areas where the conscious experience of pain occurs (Figure 2).

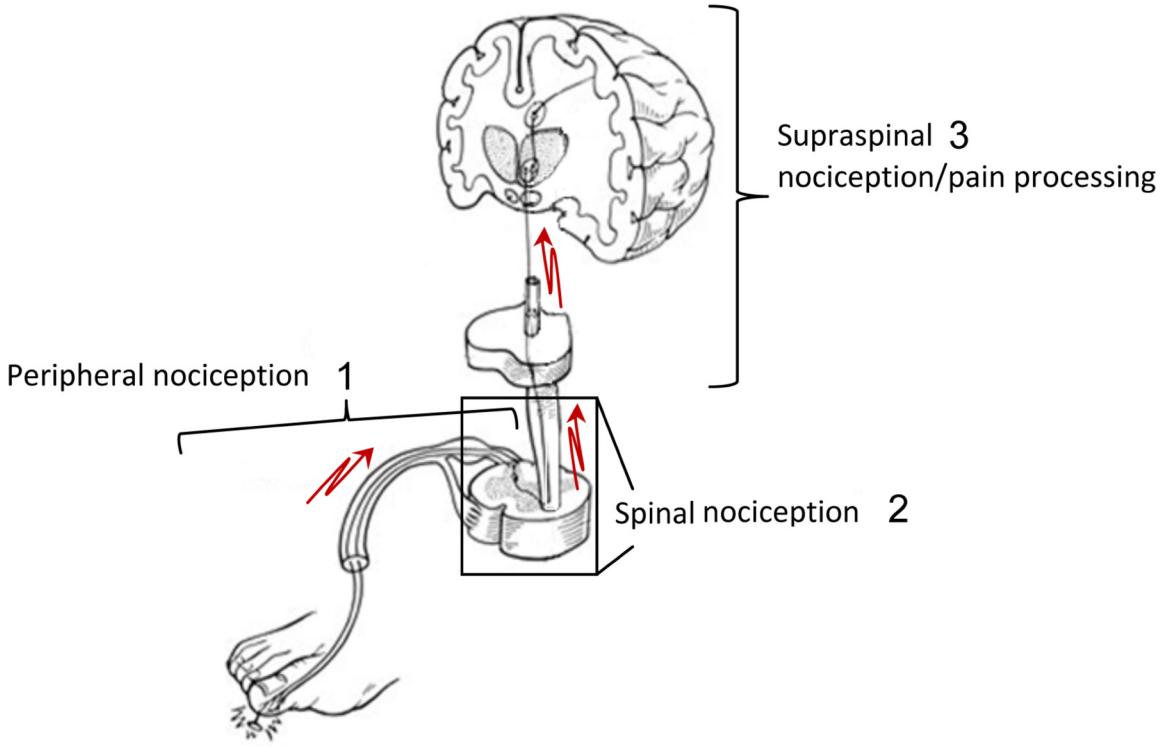

Figure 2. The Neuroaxis of pain, modified from (Fields, 2007). The conscious sensation of pain (3) is preceded by spinal nociception (2) and the peripheral nociception (1). The various parts of the nervous system are connected via nerves and fibers, and communicate electrically by sending action potentials ↯ to one another.

Although it is unknown exactly where in the brain a conscious experience occurs, the experience of pain involves the cerebral cortex and subcortical nuclei, which encode for the different sensory and emotional aspects of pain. The brain regions activated in awake humans during a painful stimulation have been studied with the use of non-invasive imaging techniques, mainly with functional magnetic resonance imaging (fMRI) (Iannetti and

Mouraux, 2010). The brain regions activated during a painful experience are collectively called the *pain matrix*. The following chapters aim to describe in detail the parts of the nervous system involved in acute and physiological pain sensation, as well as the changes that occur during chronic pain states.

Acute nociception and pain

Fibers that only respond to noxious stimuli (high threshold fibers) are called nociceptors. Most of them belong to the class of thinly myelinated *Aδ fibers* or unmyelinated *C fibers*. By contrast, innocuous sensations such as light touch and proprioceptive information are conveyed by myelinated *Aβ fibers*. Exposure to noxious stimuli *depolarizes* the primary nociceptor terminals through the opening of ion channels (Waxman and Zamponi, 2014), such as the temperature-sensitive transient receptor potential vanilloid 1 (TRPV1). If the activation is strong enough, voltage-gated sodium channels (such as $Na_V1.8$) open and elicit an action potential that propagates along the nerve fibers from their sensory nerve terminals to the *dorsal horn* of the spinal cord or the trigeminal nucleus. On their path along the sensory fibers action potentials also invade the cell bodies of the sensory nerves located in the *dorsal root ganglia* (DRG) and *trigeminal ganglia*. In the dorsal horn of the spinal cord, the central terminals of primary afferent nerve fibers form chemical synapses with second order spinal neurons, where they release the excitatory neurotransmitter L-glutamate into the synaptic cleft. The peripheral fibers terminate in various grey matter segments of the spinal cord, *Rexed's laminae*, named after the Swedish neuroscientist Bror Rexed who stained transverse spinal cords from cats and kittens with toluidine blue in 1952 (Rexed, 1952), see Figure 3.

The different nociceptive fibers have distinct properties. C fibers are unmyelinated and therefore propagate action potentials more slowly (0.5-2 m/s) than the thinly myelinated Aδ fibers (12-30 m/s) to the spinal cord (Markopoulos, 2010). Aδ fibers account for the sharp and localized pain sensation while C fibers elicit a slower, dull and more diffuse sensation. By use of various biomarkers, C fibers are further subdivided into peptidergic (calcitonin gene related peptide, CGRP, or Substance P, SP, positive) and non-peptidergic (isolectin B4, IB4 binding) C fibers. By genetically silencing these fiber subgroups, their specific roles in pain perception have been investigated. A recent study found that by ablating the peptidergic C fibers positive for the CGRPα (but not the CGRPβ) peptide resulted in heat and itch sensory deficits (McCoy et al., 2013).

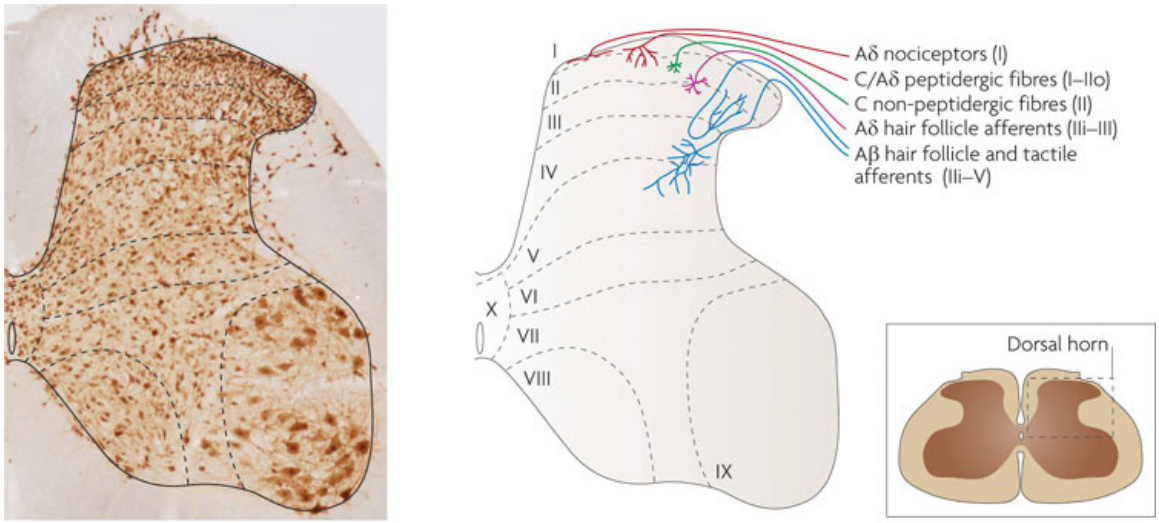

Figure 3. Pain pathways in the dorsal horn of the spinal cord, adapted from (Todd, 2010).
Left image shows a transverse cross section of half of the lumbar spinal cord stained with the neuronal marker NeuN. Right section shows the innervation coming in from the periphery. The peripheral fibers terminate in various parts of the dorsal spinal cord, subdivided in lamina I-X. Insert shows overview of the entire spinal cord cut transversely.

Interestingly, the authors also found that the mice became hypersensitive to cold, a common symptom in patients suffering from chronic neuropathic pain. Similarly, the non-peptidergic group of afferents have been implicated in mechanical (Bogen et al., 2008) and heat (Malin et al., 2006) hyperalgesia, as well as in the phenomenon of hyperalgesic priming, a persistent increased susceptibility to sensitization by inflammation (Joseph and Levine, 2010).

Nociceptors are further classified depending on the channels they express and, accordingly, on the sensory quality that excites them. For instance, sensitivity to heat partially depends on the transient receptor potential channels of the vanilloid type 1 and 2 (TRPV1/2), cold sensing comes from transient receptor potential subfamily M member 8 (TRPM8) and/or TRPA1, and several chemical irritants by the transient receptor potential subfamily A member 1 (TRPA1) (Julius and Basbaum, 2001). Primary sensory neurons also differ in their expression of vesicular glutamate transporter subtypes (VGLUTs) 1-3 (Ma, 2014), although substantial overlap of VGLUT isoform expression can occur in a single neuron. VGLUT1 is expressed in about 40% of DRG neurons including most proprioceptors and low-threshold myelinated mechanoreceptors. Roughly 90% of DRG neurons express VGLUT2 including those responsive to pain, itch, thermoception, mechanoception. VGLUT3 is expressed in about 15% of DRG neurons, probably representing low threshold unmyelinated mechanoceptors that form the lanceolate endings around hair follicles in the skin. The VGLUT3 positive neurons express neither IB4 nor CGRP, and VGLUT3 was not found in myelinated mechanoceptors.

1.1.2 Spinal pain processing

The primary afferent sensory fibers including nociceptive fibers project from the periphery to the dorsal horn of the spinal cord. In the dorsal horn, the different kinds of primary afferent fibers terminate in a characteristic pattern ordered in layers (Figure 4). Nociceptive fibers terminate in the most superficial laminae of the dorsal horn grey matter, whereas myelinated fibers terminate mainly in the deep dorsal horn. The ventral horn harbors interneurons and motor neurons that control movement and reflexes (Barry et al., 2014).

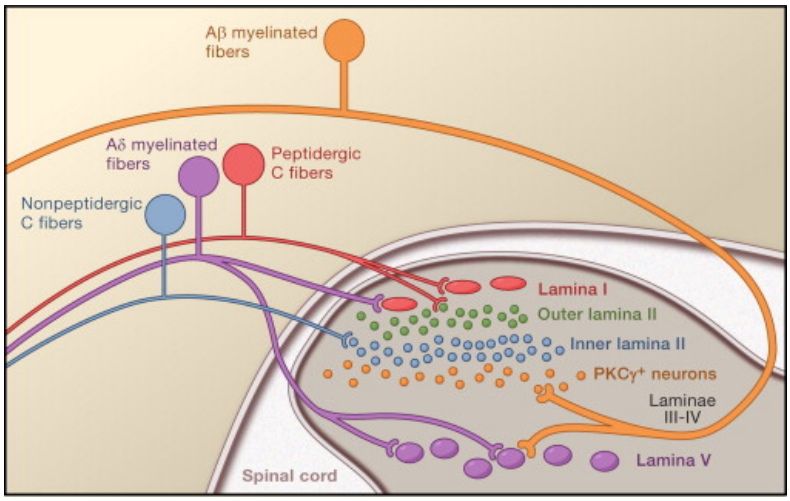

Figure 4. How primary afferents terminate in the dorsal horn of the spinal cord, adapted from (Basbaum et al., 2009).
The nociceptive information from the periphery travels along the primary afferent nerve fiber and terminates in an ordered manner in the dorsal horn of the spinal cord.

Antisera against CGRP for labelling peptidergic fibers and other markers (such as IB4 which binds to non-peptidergic fibers) have been used to describe the spinal terminals of primary nociceptive fibers in more detail in co-expression studies (Figure 5). The thickly myelinated non-nociceptive Aβ fibers are labelled in the deeper laminae with antibodies detecting the 200 kDa neurofilaments protein NF200 (Belkouch et al., 2014). They can also be identified by expression of VGLUT1. In addition, myelinated fibers take up the cholera toxin B subunit (CTB) which is transported transganglionically along the peripheral nerve and the dorsal root to the spinal cord where the fibers can then be detected with antibodies against CTB (Figure 5).

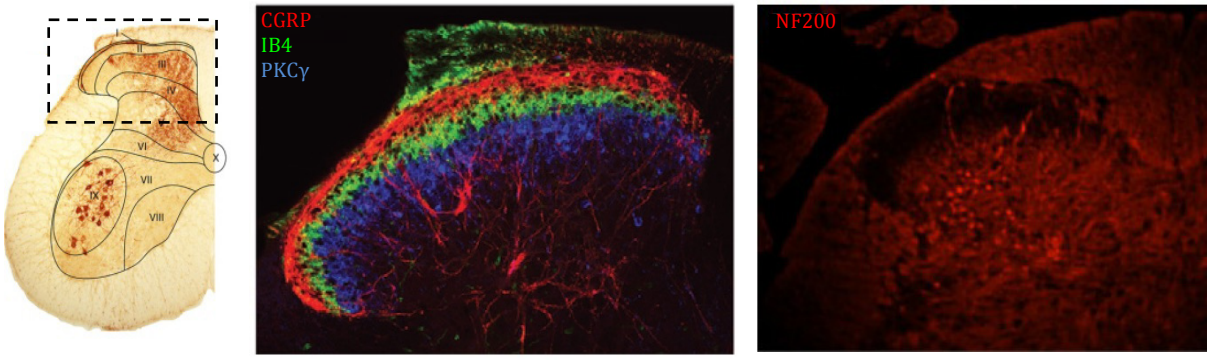

Figure 5. Distribution of primary afferent terminals in the dorsal horn of the rat spinal cord, modified from (Polgár et al., 1999; He et al., 2014; McCoy et al., 2014).
Left image shows an overview of the Rexed lamina from a mouse whose sciatic nerve was injected with the cholera toxin B subunit to label axons and terminals of myelinated sensory nerve fibers in the dorsal horn (boxed area) and motorneurons in the ventral horn. The middle image shows a triple staining using a CGRP antibody to detect peptidergic terminals in the dorsal horn in red, together with Alexa488-conjugated IB4 to detect non-peptidergic terminals in green and an antibody against PKCγ in blue. CGRP corresponds to lamina I and dorsally superficial lamina II (II outer, IIo). IB4 termination is seen in deeper lamina II (II inner, Iii). PKCγ immunoreactivity is mainly observed in the deeper lamina IIi to III. The right image shows a staining for NF200, which labels the myelinated Aβ fibers, with signal confined to the deeper laminae beyond lamina III.

The nociceptors signal to the spinal cord primarily through the release of the excitatory neurotransmitter L-glutamate. Peptidergic nociceptors release in addition substance P and CGRP into the synaptic cleft. The postsynaptic glutamate receptors include the ionotropic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), kainate and NMDA (N-methyl-D-aspartate) receptors, and the metabotropic glutamate receptors mGluR1-8 (Shigemoto et al., 1997; Ugolini et al., 1999). Substance P binds to the G-protein coupled neurokinin 1 receptor (NK-1R) (De Felipe et al., 1998) expressed in roughly 80% of spinal projection neurons (Todd et al., 2002). CGRP has been described mainly in the context of vasodilation when binding to the calcitonin gene-related peptide receptor (CGRPR) in the periphery (ter Haar et al., 2010) but it also contributes to spinal pain signaling (Gamse and Saria, 1986). Primary nociceptors can activate both excitatory and inhibitory interneurons as well as (excitatory) projection neurons directly. Figure 6 shows an example of a direct activation of a projection neuron as well as a primary afferent nerve fiber first engaging a spinal excitatory interneuron in lamina II that, through the release of glutamate, activates a lamina V projection neuron in the spinal cord.

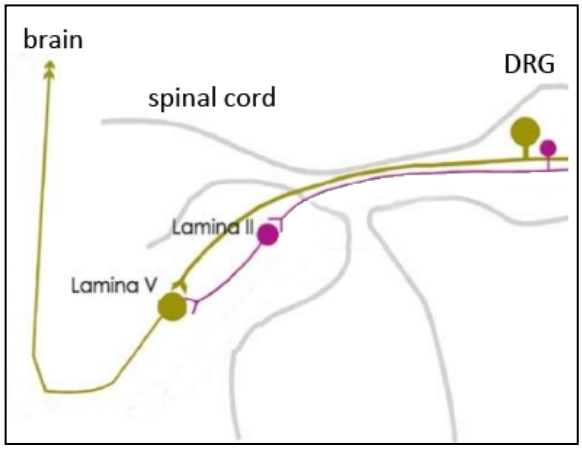

Figure 6. Monosynaptic and polysynaptic pathways in the dorsal horn of the spinal cord, adapted from (Gu and Heft, 2004).
Primary afferent input can either engage a projection neuron in the spinal cord directly as in green, or indirectly via an excitatory interneuron as in blue. Inhibitory interneurons also exist, and play a role in modulating the activity of dorsal horn neurons.

Pathological and chronic pain

It is estimated that 20 % of the European population suffer from pain lasting longer than 6 months, which is the common definition of chronic pain (Breivik et al., 2006). Sixty percent of these patients report that they are at least sometimes unsatisfied with their pain therapy. In many cases, the suffering caused by chronic pain is so severe that it leads to depression and inability to work. In the USA, the Institute of Medicine states that chronic pain affects about 100 million adults (about 25%), which is more than those suffering from cancer, diabetes and heart disease combined (Scholz and Woolf, 2002). The annual cost to society has been estimated at approximately $ 635 billion in medical treatment and loss of productivity.

General hallmarks of chronic pain syndromes include spontaneous pain felt in the absence of sensory stimulation, hyperalgesia (exaggerated pain sensations felt in response to stimuli which are already felt as painful under normal conditions) and allodynia (normally innocuous stimuli such as light touch or brushing can be felt as painful) (Ringkamp et al., 2013). The transition from acute to chronic pain can potentially come from changes occurring at the level of the primary afferent fiber, in the spinal cord or come from supraspinal alterations. Chronic pain is typically classified as inflammatory pain or neuropathic pain. Both of these pain forms differ not only in their initial cause but also in the underlying pathomechanisms.

Inflammatory pain occurs by definition in the course of chronic inflammation. A typical example is rheumatoid arthritis during which immune cells infiltrate the affected joints (Neogi and Felson, 2013), and together with cells, such as keratinocytes and mast cells, release large amounts of proinflammatory cytokines (Ji et al., 2014). These cytokines do not

only sustain and amplify inflammation but also sensitize nociceptors. Inflammatory pain is in fact thought to be to a large extent due to this sensitization of primary nociceptor terminals. Important mediators of this sensitization are arachidonic acid metabolites, such as prostaglandins, and nerve growth factor that sensitize nociceptors for hours, days or even longer. Resident cells, such as keratinocytes and mast cells, also release inflammatory mediators, including prostaglandins, protons, ATP, bradykinin, nerve growth factor (NGF) and interleukins. A well-known and tangible example of this kind of nociceptor sensitization is through NGF, which activates the tyrosine kinase receptor A (TrkA). For instance, during inflammation or in a sunburned area of the skin, NGF mRNA is increased in skin cells (such as keratinocytes) and the protein is subsequently released from cells locally to active TrkA located on nociceptors in the skin (Anand, 1995). TrkA activation initiates several signaling cascades involving second messengers. These second messengers in turn activate various kinases known to be involved in causing nociceptor hypersensitivity. This combination of inflammatory molecules is sometimes referred to as *inflammatory soup*. Some authors classify inflammatory pain and acute pain as "nociceptive pain" because both arise from the activation of nociceptors. It should however not be forgotten that inflammation also leads to the production to proinflammatory mediators in the central nervous system and that these centrally produced mediators cause changes in the central processing of nociceptive stimuli (Samad et al., 2002; Neogi and Felson, 2013). In this sense, the mechanisms of chronic inflammatory pain differ significantly from those of acute pain. Cellular signaling cascades of peripheral nociceptor sensitization are summarized in Figure 7.

Neuropathic pain originates by definition from a primary damage or dysfunction of the somatosensory system (Scadding and Koltzenburg, 2013). Typical causes of neuropathic pain include metabolic diseases (diabetes mellitus), injuries (amputations, plexus lesion), infections (postherpetic neuralgia, HIV neuropathy), exposures to toxins (ethanol, arsenic, thallium etc), and genetic defects. In some patients, the cause of their neuropathic pain remains unknown. Neuropathic pain can also originate from damage to central neurons, e.g. in patients with brain infarction or spinal cord injury. Important pathomechanisms of neuropathic pain include ectopic spontaneous action potential firing in peripheral and central neurons of the somatosensory system and the formation of abnormal functional connections between neuronal pathways usually processing distinct sensory modalities. It is meanwhile well established that peripheral nerve damage induces changes in the central nervous system, which also contribute to neuropathic pain. Activation of microglia in response to peripheral

nerve damage and subsequent disturbance of chloride homeostasis are meanwhile well-established mechanisms (Beggs and Salter, 2013).

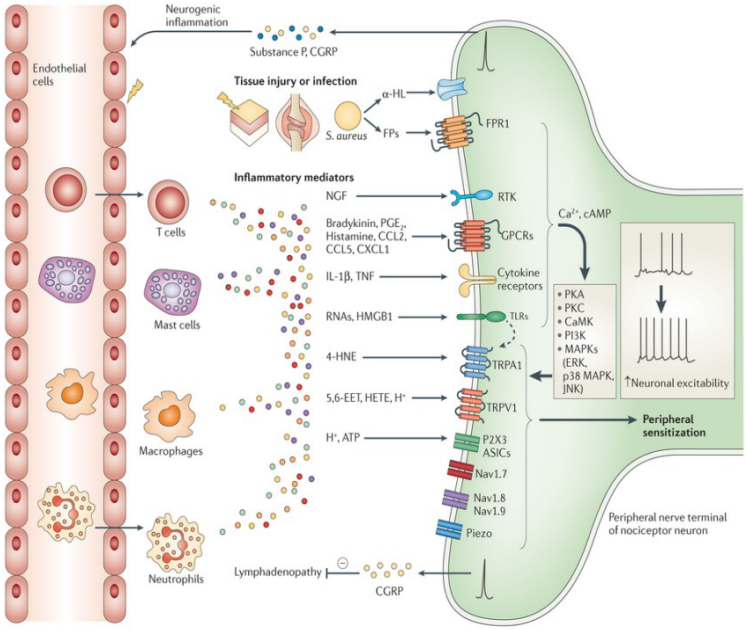

Figure 7. Molecules involved in peripheral sensitization (Ji et al., 2014).
Resident skin cells and infiltrating immune cells locally contribute to the inflammatory soup that sensitizes nociceptors. G protein coupled receptors activate kinases, such as protein kinase A and C, phosphoinositide 3-kinase, calcium/calmodulin-dependent protein kinase, and the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase, p38 MAPK and JUN N-terminal kinase. These kinases phosphorylate proteins that affect the excitability of the nociceptor, such as sodium channels, transient receptor potential family channels and Toll-like receptors. The result is a heightened sensitivity of the peripheral terminal of the sensory fiber to stimuli which cause depolarization, such as modest pressures or temperature differences. 4-HNE: 4-hydroxynonenal, 5,6-EET: 5,6-epoxyeicosatrienoic acid, FPR1: formyl peptide receptor 1, HETE: 5-hydroxyeicosatetraenoic acid, HMGB1: high mobility group protein B1, P2X3: P2X purinergic receptor 3, PGE_2, prostaglandin E_2: RTK, receptor tyrosine kinase.

It should however be kept in mind that the distinction between inflammatory and neuropathic pain is not always completely strict. Tumor pain and pain in patients with multiple sclerosis for example shares characteristics of inflammatory and neuropathic pain (Yousefzadeh et al., 2015).

Spinal sensitization

The processes that cause pain hypersensitivity and persistence can originate not only in the periphery but also in the central nervous system. This *central sensitization* (Woolf, 1983) can occur in the spinal cord and in supraspinal centers (Latremoliere and Woolf, 2009). A

symptom characteristic of central sensitization is secondary hyperalgesia. This term refers to a form of sensitization, which occurs outside an area of inflammation or nociceptor activation. It is frequently studied in response to peripheral nociceptor activation by intracutaneous capsaicin injection (Magerl et al., 1998). This intracutaneous capsaicin injection causes an acute painful sensation through TRPV1 activation at the site of injection with an accompanying flare response (reddening of the skin) and a subsequent heat and mechanical sensitization at the site of injection. Subsequent to this primary hyperalgesia, an area of secondary hyperalgesia occurs with no flare reaction and solely mechanical hypersensitivity (Meyer et al., 2006). This secondary hyperalgesia is not accompanied by an increased sensitivity of nociceptive fibers but caused by changes in central processing. It is believed that pain sensations evoked by mechanical stimulation in the secondary hyperalgesic area are elicited by activation of low threshold mechanosensitive fibers. A large body of evidence indicates that central sensitization also occurs in many clinically relevant chronic pain conditions.

Activity dependent forms of central sensitization

Intense nociceptive input to the spinal cord can induce long-lasting pain sensitization in the absence of inflammation or neuropathy. The underlying cellular and synaptic processes probably include an increase in excitability of spinal cord neurons or diminished inhibitory pain control in the spinal cord (Woolf, 1983).

Increased synaptic excitation

Intense C fiber input to lamina I projection neurons induces a form of synaptic plasticity which is remarkably similar to long-term potentiation (LTP) in the hippocampus (Sandkühler et al., 1997). Hippocampal LTP has first been described by Terje Lømo in 1966 (Bliss and Lømo, 1966) and is thought to be a cellular and synaptic correlate of learning and memory formation. In the spinal cord dorsal horn, LTP may contribute to exaggerated pain sensations (in particular hyperalgesia) following injury or inflammation.

Dorsal horn LTP can be experimentally induced in lamina I projection neurons at nociceptive synapses expressing NK-1R by intense C fiber stimulation (Ikeda et al., 2003). This spinal LTP is abolished by blockade of NK-1R, NMDA receptors or low threshold voltage-gated calcium channels (Ikeda et al., 2006). Depending on the type of projection neuron (parabrachial or periaqueductal grey) LTP required either high or low stimulation frequencies. Recent work by Bonin & De Koninck confirmed the remarkable similarities

between spinal pain sensitization process and hippocampal memory formation through LTP (Bonin and De Koninck, 2014). Spinal LTP observed after intraplantar capsaicin injection and, like hippocampal LTP, depends on protein synthesis and has the potential to be extinguished if re-activated (by a second capsaicin injection) paired with protein synthesis inhibition.

Diminished synaptic inhibition

Well-balanced excitation and inhibition in the spinal nociceptive circuits is necessary to preserve their physiological functions and to keep them quiescent during innocuous inputs. The inhibitory interneurons rely on their release of GABA and glycine to evoke inhibition of the postsynaptic cells, which express ionotropic $GABA_A$ and strychnine-sensitive glycine receptors. Through the central pore of the activated $GABA_A$ and glycine receptors, negatively charged Cl^- ions flow through the cell membrane and reduce neuronal excitation through *hyperpolarization* and activation of a shunting conductance.

The first experiments implicating a GABAergic or glycinergic tone modulating nociceptive processes at the level of the spinal cord were performed by spinal application of the glycine receptor antagonist strychnine at sub-convulsive concentrations in rats (Beyer et al., 1985). These rats exhibited reoccurring grooming, biting and licking behavior as well as vocalization responses to light cutaneous stimulation. Similar results were obtained after spinal application of the $GABA_A$ receptor antagonist bicuculline (Sivilotti and Woolf, 1994). Subsequent studies could show that strychnine and bicuculline exaggerate the pain sensitivity seen in rats after sciatic nerve constriction (Yamamoto and Yaksh, 1993). A recent publication demonstrates that targeted unilateral silencing or ablation of dorsal horn glycinergic interneurons evoked thermal and mechanical hyperalgesia as well as signs of spontaneous discomfort (Foster et al., 2015).

On the cellular level, loss of GABAergic inhibition in the dorsal horn causes Aβ fiber input to excite spinal nociceptive neurons. Activity of lamina II neurons was recorded before and after an application of the $GABA_A$ receptor antagonist bicuculline (Figure 8). Under control condition, Aβ fiber intensity had only minor effects, while in the presence of bicuculline, Aβ fiber stimulation elicited hyperexcitability (Baba et al., 2003). This suggests that loss of GABAergic inhibition in the spinal cord can turn normally innocuous stimuli into painful stimuli. Similar observations can be made after block of inhibitory glycine receptors (Sivilotti and Woolf, 1994).

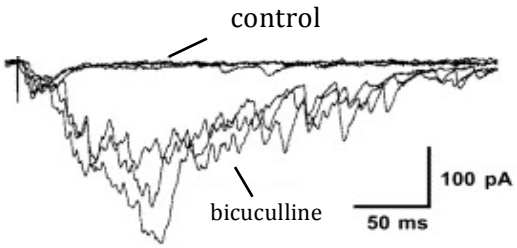

Figure 8. Aβ fiber stimulation in the absence (control) and presence of bicuculline, modified from (Baba et al., 2003).
Electrophysiological patch clamp recordings from lamina II of lumbosacral rat spinal cords. Stimulation of the dorsal root at Aβ fiber intensity usually evokes fast mono- or polysynaptic excitatory postsynaptic currents with short duration (50–200 ms, se control traces). After 40 μM bath application of bicuculline, Aβ fiber intensity stimulation reproducibly causes long-lasting (300–1000 ms) polysynaptic excitatory postsynaptic currents.

Research from the last 15 years has shown that GABAergic and glycinergic inhibition can get compromised through a variety of different mechanisms including posttranslational changes in neurotransmitter receptor function, regulation of neurotransmitter synthesis and changes in the intracellular concentration of chloride (Zeilhofer et al., 2009).

The inflammatory mediator prostaglandin PGE_2 – but not the prostaglandins PGI_2, $PGF_{2\alpha}$ or PGD_2 – causes disinhibition in spinal dorsal horn neurons (Ahmadi et al., 2002) through reduced responsiveness of superficial dorsal horn glycine receptors, which occurs through a PGE_2- and EP2 receptor-dependent activation of protein kinase (PK) A acting on the glycine receptor subunit α3 (Figure 9). Mice lacking this subunit show reduced sensitization by spinal PGE_2 application or by peripheral inflammation (Harvey et al., 2004).

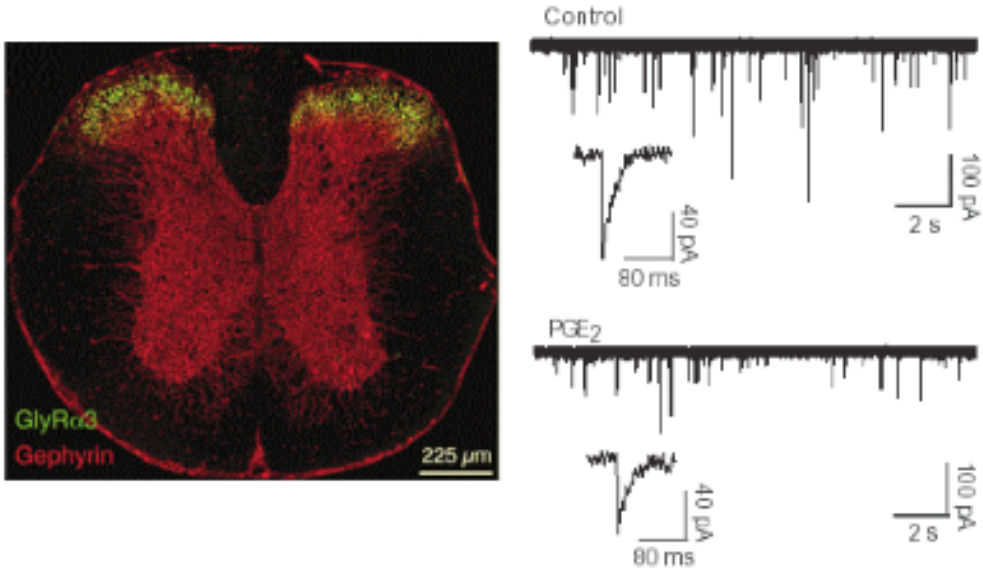

Figure 9. Spinal expression pattern of α3 glycine receptors and modulation by PGE_2 from (Harvey et al., 2004) and (Ahmadi et al., 2002).
Recordings are from superficial rat dorsal horn neurons. Top shows recordings from untreated rat lumbar spinal cord slices. Bottom shows recording during treatment with PGE_2, which leads to a reduction in mIPSC amplitude but not frequency.

Following peripheral nerve injury, the potassium chloride co-exporter 2 (KCC2) is down-regulated in a process that is dependent on brain derived neurotrophic factor (BDNF) released from activated (ATP-stimulated) microglia (Coull et al., 2005) (Figure 10). This KCC2 down-regulation causes a disruption of the transmembrane chloride gradient in superficial dorsal horn neurons. This ensuing hyperalgesia that can be mimicked in healthy animals by spinal application of KCC2 blockers or silencing KCC2 RNA (Coull et al., 2003).

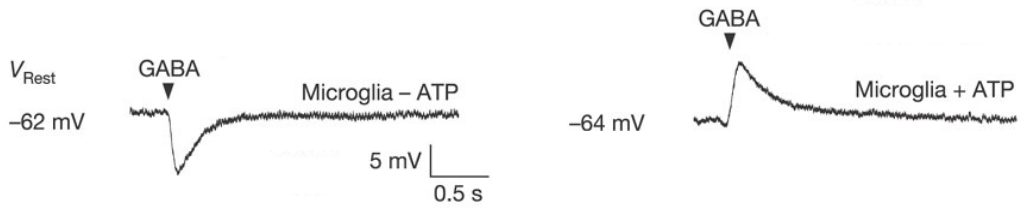

Figure 10. GABA becomes depolarizing in the presence of ATP-stimulated microglia, modified from (Coull et al., 2005).
Left shows lamina I neurons from control mice without ATP-stimulated microglia (Microglia – ATP) exhibiting a hyperpolarizing effect of GABA application on the cellular voltage at resting membrane potentials. Same experiment repeated with ATP-stimulated microglia shows a depolarizing effect of GABA application at resting membrane potentials.

In 2011, Zhang et al. showed that epigenetics are implicated in chronic pain. During inflammation and nerve injury, the GABA synthesizing enzyme GAD65 is down-regulated in the brainstem by epigenetic changes (Zhang et al., 2011). The histone deacetylase (HDAC)-mediated histone hypoacetylation suppressed the expression of the gene *Gad2*, encoding the protein GAD65, leading to disinhibition in the nucleus raphe magnus. HDAC inhibitors were effective in reversing the hyperalgesia. A down-regulation of GAD65 has also been reported for the spinal cord (Moore et al., 2002; Lorenzo et al., 2014).

Structural changes

In addition to the function changes described above, structural changes in dorsal horn circuits have also been proposed to contribute to pain sensitization. Inflammation induced by peripheral injection of complete Freund's causes Aβ fibers to sprout into the superficial laminae of the spinal cord (Ma and Tian, 2002). As a result, innocuous stimulation would activate superficial nociceptive dorsal horn neurons. The extent to which this plasticity contributes to chronic pain is controversial. Recent reports have not found Aβ fiber sprouting to occur in spite of observable allodynia, for instance after nerve injury (Hughes et al., 2003; Shehab et al., 2004).

Peripheral nerve damage may also induce the death of dorsal horn inhibitory interneurons (Moore et al., 2002; Scholz et al., 2005; Meisner et al., 2010), but others have provided data

suggesting that cell death in the dorsal horn is not required for the development of neuropathic hyperalgesia (Polgár et al., 2004; Polgár et al., 2005; Todd, 2010).

Supraspinal pain processing

From the spinal cord, neurons project up towards the brain along several different ascending tracts (Figure 11). These include the spinothalamic, spinoreticular, spinoparabrachial, spinomesencephalic, spinoolivary, spinohypothalamic, spinoamygdalar and spinocerebellar pathways (Mai and Paxinos, 2012). The most prominent ones are the spinothalamic tract (projecting to the thalamus) and the spinoreticular tract (projecting to the brainstem). These various projections together make up the three dimensions of pain sensation that were suggested by Melzack and Casey in 1968 (Melzack and Casey, 1968):

- *sensory-discriminative*: sensing the intensity, location, quality and duration of the pain, involving the brain areas thalamus, primary and secondary somatosensory cortex (Auvray et al., 2010)
- *affective-motivational*: unpleasantness and urge to escape the unpleasantness of the pain, involving brain areas such as anterior cingulate cortex and amygdala (Gao et al., 2004)
- *cognitive-evaluative*: cognitions such as appraisal, evaluation of pain, cultural values, distraction and hypnotic suggestion of the pain, with activity seen during fMRI even in absence of painful stimuli during evaluation tasks in insular and prefrontal cortex (Kong et al., 2006)

Melzack and Casey predicted that the experience of pain is not only described by its intensity or magnitude of stimulation, but also is influenced by psychological factors and the environmental context (Geva and Defrin, 2013; Petersen et al., 2014).

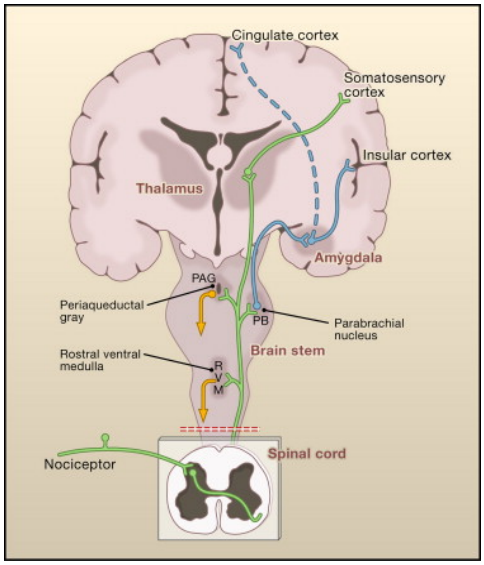

Figure 11. The neuroaxis of pain and its various relay stations on the way up to the brain, from (Basbaum et al., 2009).
A "painful" peripheral stimulus activates the primary afferent nerve fiber (nociceptor) which terminates in the dorsal horn of the spinal cord. The termination is a chemical synapse within which glutamate is released to activate the postsynaptic spinal neuron. In this monosynaptic example, the postsynaptic neuron is a projection neuron. The axon of the projection neuron crosses the midline and proceeds rostrally up to the brain along the spinothalamic, spinoreticular or spinoparabrachial pathway – although spinal projections to other areas of the brain also occur. In the example of the thalamus, the spinothalamic axons forms another chemical synapse, where the postsynaptic target is a thalamic neuron that projects to many higher centers in the brain, such as the primary and secondary sensory cortex or anterior cingulate gyrus and cingulate cortex. Not only does the spinal cord serve as a runway for ascending pain information, but also descending pain inhibition. From the brainstem nucleus rostroventromedial medulla and periaqueductal grey, fibers descend back down to the dorsal horn to inhibition pain transmission form reaching the brain.

Descending modulation of dorsal horn pain processing

Different *descending fibers* from the brain onto the dorsal horn of the spinal cord have the capacity to either increase and decrease spinal nociception (Gebhart, 2004). This top-down modulation is mainly mediated by descending monoaminergic pathways from various supraspinal sites that either inhibit or facilitate transmission of nociceptive information at the level of the dorsal horn (Millan, 2002) (see Figure 12).

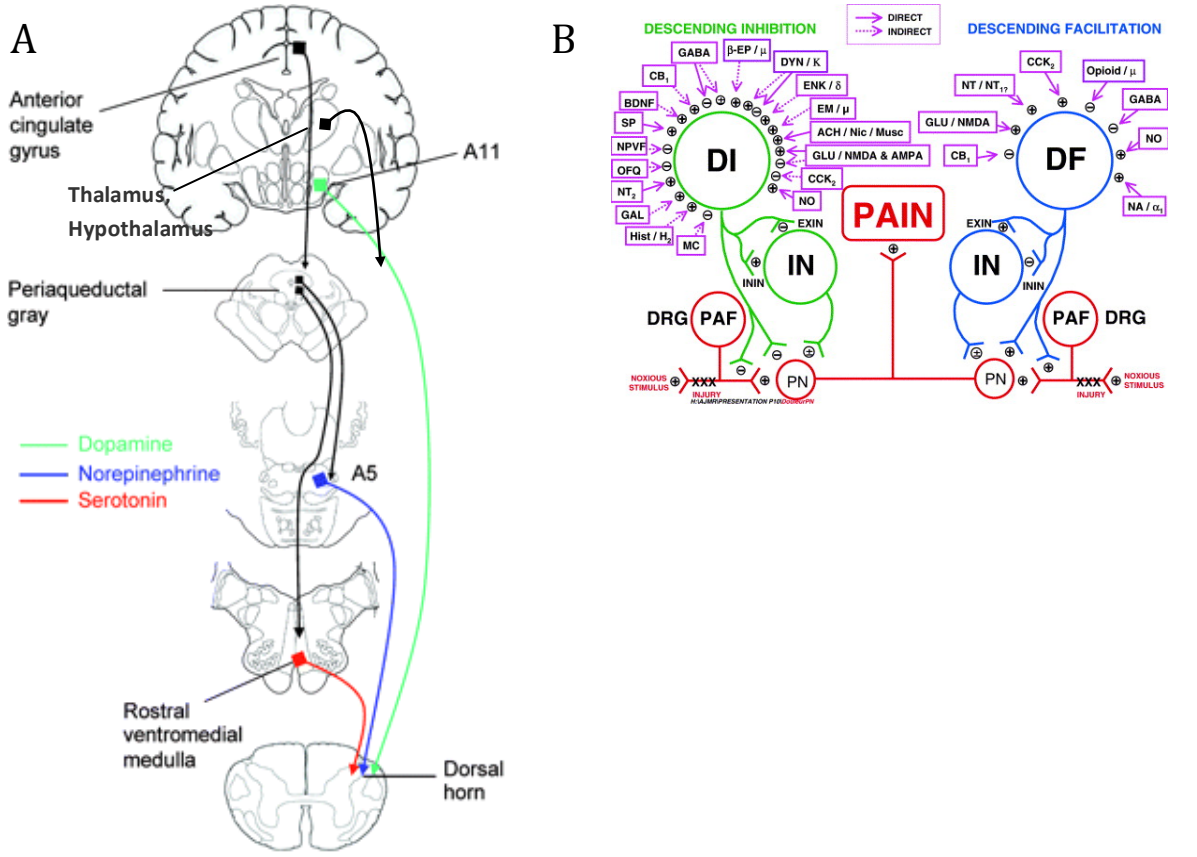

Figure 12. Descending modulation of pain, from (Benarroch, 2008) and (Millan, 2002).
(A) Descending monoaminergic pathways. (B) Overview of described molecules involved in descending inhibition (DI) and facilitation (DF) acting directly or indirectly on dorsal horn neurons. At the dorsal horn, pathways mediating DI and DF have opposite effects on primary afferent fiber (PAF) terminals, projection neurons (PN), inhibitory interneurons (ININ) and excitatory interneurons (EXIN). DRG, dorsal root ganglion; NO, nitric oxide; CCK, cholecystokinin; NT, neurotensin; GLU, glutamate; NMDA, *N*-methyl-d-aspartate; ACh, acetylcholine; musc, muscarinic; nic, nicotinic; DYN, dynorphin; ENK, enkephalin; β-EP, β-endorphin; EM, endomorphin; GABA, γ-hydroxy-butyric acid; Hist, histamine; CB, cannabinoid; NA, noradrenaline; SP, substance P; NPVF, neuropeptide VF and OFQ, orphanin FQ (nociceptin)

The periaqueductal grey (PAG) has been recognized for over half a century for its ability to cause analgesia after being electrically stimulated (Reynolds, 1969). Subsequent studies revealed the ventrolateral PAG as the main projection area responsible for analgesia through descending inhibition (Yaksh and Rudy, 1978). More recently, it was discovered that stimulation in many sites upstream of the PAG in the brain, including the somatosensory cortex, hypothalamus, thalamus, as well as midbrain, pons and medulla structures could produce inhibitory effects on spinal nociceptive processing (Gebhart, 1986; Hammond, 1986). A particularly important relay station downstream of the PAG was shown to be the rostroventromedial medulla (RVM). Antinociception seen after PAG-stimulation could be abolished when the RVM was inactivated by local injection of the sodium channel blocker lidocaine (Gebhart et al., 1983). Today, many more descending pathways have been described, involving the cannabinoid and opioid system.

Supraspinal pain sensitization

The spinal activity-dependent LTP or long term depression (LTD) can also manifest through prolonged activity reaching the supraspinal pain processing sites. The consequences can similarly be a dysfunctional and hyperexcitable pain circuit and can potentially explain the various comorbidities often seen in patients suffering from chronic pain, such as anxiety and depression (Yalcin et al., 2014). Epidemiological studies reveal that around 50% of chronic pain patients also suffer from depressive disorders (Kroenke et al., 2011).

Likely, different brain regions will be involved in supraspinal sensitization, depending on the type of pain disorder. Anatomically, the brain is more complex than the spinal cord and molecular changes in one nucleus can affect associated brain regions that together cause a persistent pain state. A time-dependent increase in descending facilitation from the RVM have been shown to maintain neuropathic pain states (Burgess et al., 2002). After spinal nerve ligation, lidocaine injected into the RVM reverses thermal hypersensitivity one week after nerve injury, but is ineffective three days after injury. The authors also found that lesioning the descending dorsolateral funiculus prevented the supraspinally-induced increase of dynorphin after spared nerve injury, and also blocked maintenance of the hyperalgesia. Excitatory descending input to the spinal cord is mediated by serotonin released from brainstem neurons (Suzuki and Dickenson, 2005). Reports have shown that ondasterone, the antagonist of $5HT_3$ receptors, has efficacy against mechanical pain after peripheral nerve injury (Suzuki and Dickenson, 2005). A study on human patients with chronic neuropathic pain showed that block of $5HT_3$ receptors with ondasterone could significantly reduce pain scores compared to placebo (McCleane et al., 2003). Post-stroke pain is a form of untreatable pain that develops after a brain stroke episode. Although poorly understood, animal models such as that of experimental thalamic hemorrhage by unilateral intra-thalamic collagenase IV injection have helped to describe its features (Yang et al., 2014).

CURRENTLY AVAILABLE ANALGESICS

Several classes of analgesics are available today with distinct modes of action and clinical indications. These compounds mainly fall into two categories, cyclooxygenase (COX) inhibitors and opioids. Some forms of chronic pain, in particular neuropathic pain respond better to anticonvulsants or antidepressants than to classical analgesics.

Non-steroidal anti-inflammatory drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen and naproxen, inhibit the cyclooxygenase enzymes (COX-1 and COX-2) that convert arachidonic acid into prostaglandin precursors. Prostaglandins serve important functions in body homeostasis but are also mediators of pain and inflammation. COX-2 expression is induced in many tissues by pro-inflammatory cytokines and leads to subsequent increases in tissue levels of prostaglandins to further promote inflammation, to sensitize peripheral nociceptors and spinal nociceptive processing, and to induce fever through a hypothalamic site. Inhibition of prostaglanin synthesis by NSAIDs therefore reduces inflammatory hyperalgesia and fever and dampens symptoms of inflammation (Day and Graham, 2013). Prostaglandins involved in body homeostasis originate mainly from COX-1. Typical side-effects of classical non-selective COX-1/2 inhibitors include gastric ulcer formation and kidney damage. The more recently developed COX-2 selective inhibitors such as celecoxib, etoricoxib and rofecoxib (the latter has been withdrawn from the market in 2004) have shown analgesic efficacy with reduced risk of gastric bleeding (Derry and Moore, 2013). Both non-selective NSAIDs and COX inhibitors increase the risk of cardiovascular events. Paracetamol is a mild analgesic with fever reducing properties but no anti-inflammatory action. Several mechanisms of action have been suggested, including COX-2 inhibition (Hinz et al., 2008) and cannabinoid receptor modulation (Högestätt et al., 2005). Paracetamol causes fewer side effects but an overdose can be lethal due to liver toxicity. While commonly used for treatment of mild and inflammatory pain, these drugs are not effective in pain disorders that manifest independently of prostaglandin production, such as neuropathic pain.

Opioids

Opioids (such as morphine) are agonists at opioid receptors, which are G protein coupled receptors that, in general, lead to inhibition of synaptic transmission and neuronal excitability. In the pain pathways, opioids have two major sites of action. In the spinal dorsal

horn, they inhibit the release of excitatory neurotransmitters from primary nociceptor terminals in the spinal cord. In the brain stem, opioids enhance the activity of endogenous pain control by disinhibiting antinociceptive fiber tracts that descend form the brainstem to the dorsal horn.

Opioids are highly efficacious analgesics but exhibit various side-effects such as sedation, constipation, respiratory depression, strong euphoria, opioid dependence, chronic itch (pruritus), addiction and the paradoxical opioid-induced hyperalgesia. Prolonged use also induces tolerance (i.e. a progressive loss of effect during prolonged treatment) through only incompletely understood mechanisms (Pradhan et al., 2010). Opioids are excellent drugs for the treatment of acute (traumatic or postoperative) pain and chronic malignant pain. However, their use in patients with chronic non-malignant pain is problematic mainly because of their propensity to dependence (Hermann et al., 2005). Furthermore, their efficacy in neuropathic pain patients remains controversial (McNicol et al., 2013).

Anticonvulsants and antidepressants

Compounds used to treat nervous system disorders unrelated to pain have in some cases therapeutic potential for the treatment of chronic pain. In case of neuropathic pain, such compounds include mainly antidepressants and anticonvulsants.

Antidepressants have shown potential in the treatment of various persistent pain states, including neuropathic pain, fibromyalgia, rheumatoid arthritis, low back pain and headache (Dharmshaktu et al., 2012). Drugs approved for use in at least some neuropathic pain conditions include the tricyclic antidepressant amitriptyline. Its mood-altering properties originate most likely from the inhibition of serotonin and noradrenaline reuptake. A prolonged presence of noradrenaline and serotonin in spinal cord synapses probably also underlies the efficacy of amitriptyline in neuropathic pain. More recently developed serotonergic and adrenergic antidepressants, such as duloxetine (a selective noradrenaline-serotonin reuptake inhibitor, SSNRI), are also well established in the treatment of neuropathic pain (Bril et al., 2011) and cause fewer side-effects.

Anticonvulsants and antiepileptic drugs have also shown to be effective against chronic pain, including fibromyalgia, diabetic neuropathy and neuropathic pain (Attal et al., 2010; Moore et al., 2011). Gabapentin and pregabalin are first-line treatments against neuropathic pain. They probably reduce pathologically heightened nociceptive transmission at the primary

nociceptor terminal by targeting the α2δ subunit of voltage gated calcium channels (Silverman, 2008; Celikyurt et al., 2011). While their names contain GABA, it should be noted that neither gabapentin nor pregabalin bind to $GABA_A$ or $GABA_B$ receptors.

Carbamazepine, a voltage gated sodium channel blocker (Hebeisen et al., 2015), is an anticonvulsant, mood-stabilizer as well as analgesic effective for trigeminal neuralgia and neuropathic pain, but is not recommended as a first choice analgesic due to increased risk of suicide (Yaylacı et al., 2012).

TESTS FOR MEASURING PAIN IN LABORATORY ANIMALS

As stated above, pain is defined by the IASP as a conscious sensation. Because animals cannot report their pain as humans can do, pain studies in animals have to rely on the assessment of surrogate parameters. Almost all pain tests in animals are based on the quantification behavioral (motor) read-outs. These include withdrawal responses upon exposure to acute noxious stimulation or spontaneous aversive behaviors, such as flinches of irritated paws and vocalizations, and read-outs related to conditioned place preference or aversion. Read-outs independent of motor function are mainly based on imaging or EEG techniques measuring brain activity.

Tests designed to measure pain in rodents

Withdrawal response-based measurement

Acute nociceptive pain sensitivity is often quantified according to the threshold intensity of a nociceptive stimulus that evokes a response or according to the response latency, i.e. the time interval between the onset of a defined nociceptive stimulus and the onset of the animal's response (Le Bars et al., 2001; Barrot, 2012). Nociceptive stimuli applied in such tests include electrical, chemical, thermal and mechanical stimuli.

Pain induced by electrical application is less commonly used in animal pain tests, mainly as it involves activation of all primary afferents and does not represent a naturally occurring stimulus (Barrot, 2012). The readouts are escape behavior or vocalization of the animal. Electrical pain tests are often used in human studies due to the stimuli being non-invasive and its parameters relatively easily controlled.

Sensitivity to heat can be assessed in the hot plate test (van Eick, 1967), where a mouse is placed in an enclosed chamber with the floor at temperatures of 40-50°C and the latency to respond, either by licking of the hind paws or jumping of the animal, is recorded. The Hargreaves test allows for repeated measurements by focusing a warm beam of light from below the animal, placed on a glass surface, directed to the hindpaw or tail and measuring latency to withdrawal (Hargreaves et al., 1988). Cold sensitivity is assessed either by injection of a compound that induces a cold sensation, such as icillin (Tse and Wei, 1986), or by application of a drop of acetone to the plantar surface of the hind paw (Kontinen et al., 1998). A recent publication describes the use of dry ice applied to a glass surface below the hind paw as a means to test for cold allodynia (Brenner et al., 2012).

Mechanical stimulation is most often applied from below onto the plantar surface of the hind paw of an animal situated on a metal mesh. Noxious mechanical stimulation is performed with the pin prick test, where a sharp needle that does not perforate the skin is applied to the hind paw and percentage of elicited flinching responses are calculated (Krag and Rasmussen, 1975). The Randall-Selitto apparatus allows clamping the hind paw or tail with manually controlled pressure intensity, but requires the animal to be restrained or trained prior to testing (Randall and Selitto, 1957). Non-noxious stimuli include the flexible von Frey filaments, designed over 100 years ago by Austrian-German physiologist Maximilian von Frey (Norrsell et al., 1999). Early pain tests relied on a set of calibrated filaments that applied defined pressures to the tissue, depending on the rigidity of the filament. Later, the handheld electronic von Frey apparatus allowed for using a single flexible filament for exact readouts of the pressure applied that had been applied manually (Vivancos et al., 2004). The latest dynamic von Frey applies pressure with a defined speed and acceleration and records paw withdrawal threshold automatically (Nirogi et al., 2012).

Tests involving on-going nociceptive behavior

A limitation of the tests discussed above is that they do not evaluate spontaneous responses to ongoing pain, and rely on readouts that, in most cases, involve spinal reflexes rather than supraspinal pain processing. Chemical nociception is assessed by injection of for instance capsaicin (Baraz et al., 1968), mustard oil (Wesselmann et al., 1998) or formalin (Hitchens et al., 1967), which are most often injected subcutaneously into the plantar surface of the hind paw or the tail. The subsequent readout is the amount of time spent or the number of instances of licking, biting or flinching of the injected tissue. Visceral pain is assessed by

intraperitoneal injection of acetic acid (Koster et al., 1959) or hypertonic saline (Collier and Schneider, 1969), whereafter the number of body writhing events is counted .

More recently developed alternative approaches include the facial grimace scale test, which allows a partly automated assessments of spontaneous pain by studying the facial expression of animals in pain (Sotocinal et al., 2011). The tests involve video recordings of the animals and off-line analysis of several parameters such as orbital tightening, nose and cheek flattening, changes in ear shape, and abnormal whisker orientation. Rodents in pain will also spontaneously give off ultrasound vocalizations above 20 kHz. Although reproducibly recording pain-induced ultrasound vocalizations of animals over long periods of time has proven a challenging (Wallace et al., 2005), robust readouts for up to several week have been obtained for both cancer pain and neuropathic pain when 37 and 50 kHz vocalizations were recorded (Kurejova et al., 2010).

Conditioned place preference

Another approach has been to use place preference (or place conditioning) tests as a means to quantify the analgesic properties of drugs (King et al., 2009; Stevenson et al., 2015). In this paradigm, rodents with chronic pain are placed in a cage with two identical compartments connected to each other. Naïve animals will spend equal times exploring both chambers. To assess analgesic activity of a test compound, animals in chronic on-going pain conditions will be "conditioned", i.e. they will be trained to associate one of the two compartments with pain relief. This is done by repeatedly treating the animal with an analgesic drug in one of the chambers. After this training session a conditioned animal will prefer the chamber associated with drug treatment over the other chamber. In this way, potential pain relieving efficacy can be studied based on reduction in tonic pain rather than stimuli-induced responses. An important limitation of the test is that it will produce false positive results for drugs with rewarding properties (Navratilova et al., 2013).

Functional brain imaging

As outlined above, pain is defined as a "conscious sensation" and must as such have a cellular correlate in neuronal activity measureable in the cerebral cortex or related brain structures (Apkarian et al., 2013). Functional brain imaging employing the so called blood oxygen level-dependent (BOLD) effects should hence be well-suited to capture the very essence of pain (Lassen et al., 1978). BOLD effect-based imaging of brain activity has become a core technique in human pain research. Seminal studies have led to the view that in

the brain pain is not represented in a single area but rather leads to the activation of several brain areas potentially representing the different aspects of pain (sensory versus affective etc) (Kenshalo and Isensee, 1983). These areas have collectively been called the "pain matrix" (Derbyshire et al., 1997). More recent studies have challenged this view showing that very similar brain areas can be activated by different arousing stimuli unrelated to pain (salience detection) (Legrain et al., 2011). Most recent evidence indicates that activation of parts of the insular cortex might be most closely related to the experience of pain (Barthas et al., 2015).

fMRI is also used to assess pain in laboratory animals but its use in preclinical pain research is less wide-spread than in human pain research. Nevertheless, pain imaging has been used to assess analgesic activity objectively in rodents. Figure 13 shows an example of a study in which rat fMRI was used to assess the analgesic activity of a novel benzodiazepine site ligand.

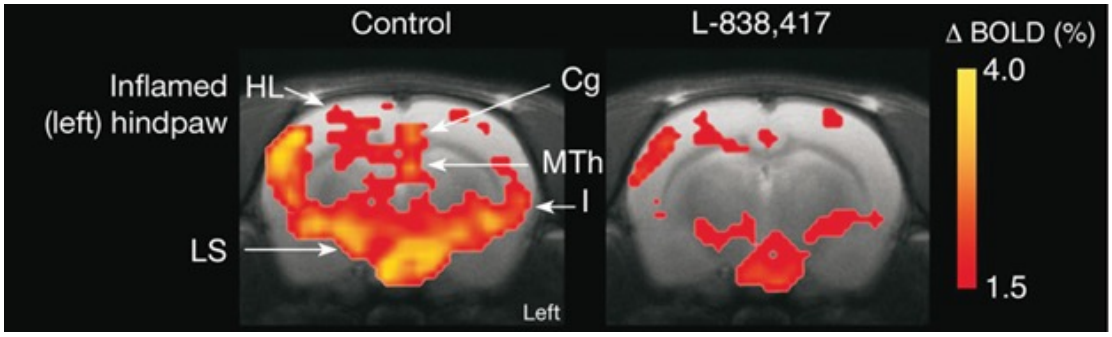

Figure 13. fMRI scans of inflamed rats at the thalamic level, treated with vehicle or 1mg/kg of L-838,417. BOLD signal in vehicle (left brain) and drug (right brain) treated rats. Key brain regions active during pain sensation had reduced BOLD signal after treatment with L-838,417. MTh, medial thalamus; S1, primary somatosensory cortex; Cg, cingulate cortex; I, insular cortex; LS, limbic system; HT, hypothalamus; HL, representation of hindlimb in S1. Left, left hemisphere.

Models of chronic pain

The majority of chronic pain models involve partial damage to peripheral nerves. The first among those was the chronic constriction injury (CCI) model (Bennett and Xie, 1988), in which several loose ligatures are placed around a peripheral nerve, typically the sciatic nerve. This procedure interferes with the blood supply of the nerve and causes progressive damage of the affected nerve fibers followed by the development of heat, cold and mechanical hyperalgesia over several days. This hypersensitivity persists for several weeks. Other more recently developed models include the spared nerve injury (SNI) (Decosterd and Woolf, 2000), spinal nerve ligation (SNL) (Kim and Chung, 1992), spinal nerve transection (SNT)

(Sheen and Chung, 1993), and partial sciatic nerve injury (PSNI) (Seltzer et al., 1990), summarized in Figure 14. Animals subjected to these surgeries recapitulate many of the symptoms observed in humans, such as touch-evoked allodynia, and thermal and mechanical hyperalgesia (Honoré et al., 2011). These neuropathic pain models produce a hypersensitivity lasting for several weeks.

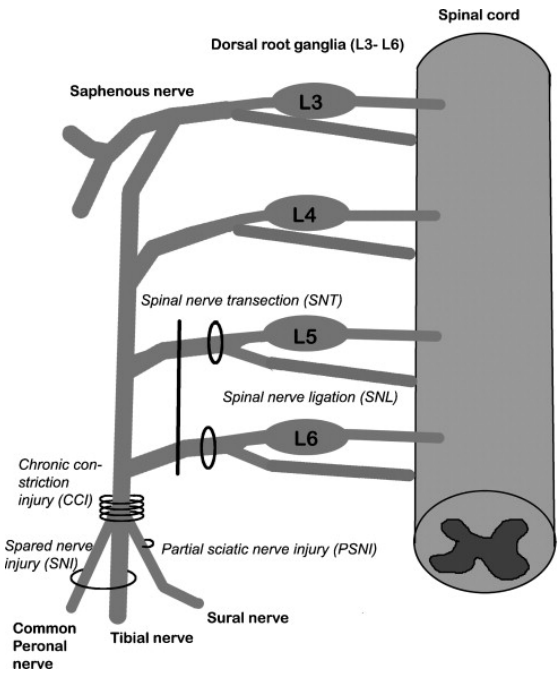

Figure 14. Surgical protocols for eliciting models of chronic neuropathic pain in rodents, from (Honoré et al., 2011).

Inflammatory pain is typically studied after injection of pro-inflammatory compounds into one paw. Frequently used compounds include the yeast β-glucan zymosan A (Doherty et al., 1985), the seaweed polysaccharide carrageenan (Rios and Jacob, 1983), and Complete or Incomplete Freund's Adjuvant (Yonehara et al., 1983).

Other models are aimed to recapitulate specific painful pathologies. Intravenous injection of streptozotocin causes diabetes and evokes a painful diabetic neuropathy within one week of injection, including thermal hyperalgesia (Forman et al., 1986). Paclitaxel (taxol) is a cytotoxic agent used in chemotherapy of different cancers. When given systemically to rodents, it causes a peripheral painful neuropathy. Cancer pain can be studied in rodents by carcinoma cell transplants injected into various bones of the hind limb (most often used are femur, tibia and calcaneus) (Medhurst et al., 2002). The animals typically develop bone-tumor and ensuing structural damage of the affected bone, accompanied with mechanical hyperalgesia within two weeks of transplantation.

The following chapter provides a comprehensive overview of the current knowledge on $GABA_A$ receptors in spinal pain control and on their potential role as targets for novel analgesic compounds.

RESTORING THE SPINAL PAIN GATE: GABA$_A$ RECEPTORS AS TARGETS FOR NOVEL ANALGESICS*

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Abstract

$GABA_A$ receptors ($GABA_ARs$) and glycine receptors (GlyRs) are key elements of the spinal control of nociception and pain. Compromised functioning of these two transmitter systems contributes to chronic pain states. Restoring their proper function through positive allosteric modulators should constitute a rational approach to the treatment of chronic pain syndromes involving diminished inhibitory spinal pain control. Although classical benzodiazepines (i.e. full agonists at the benzodiazepine binding site of $GABA_ARs$) potentiate synaptic inhibition in spinal pain controlling circuits, they lack clinically relevant analgesic activity in humans. Recent data obtained from experiments in $GABA_AR$ point-mutated mice suggests dose-limiting sedative effects of classical non-specific benzodiazepines as the underlying cause. Experiments in genetically engineered mice resistant to the sedative effects of classical benzodiazepines and studies with novel less sedating benzodiazepines have however shown that profound antihyperalgesia can be obtained at least in preclinical pain models. Present evidence suggests that compounds with high intrinsic activity at α2-$GABA_AR$ and minimal agonistic activity at α1-$GABA_AR$ should be pursued in order to achieve maximum antihyperalgesia and no unwanted sedation. On-going preclinical studies in genetically engineered mice and clinical trials with more selective benzodiazepine site agonists should soon provide additional insights into this emerging topic.

Introduction

Chronic pain is a severe medical condition affecting millions of patients world-wide. It is almost generally accepted that neuronal and synaptic plasticity occurring at different levels of the neuraxis are major contributors to chronic pain (Luo et al., 2014; Sandkühler, 2009; Zeilhofer et al., 2009b) (for a schematic illustration of the pain pathway see figure1). Some of these neuroplastic changes occur already in the peripheral terminals of nociceptors, which sense noxious stimuli arriving at the skin or in other peripheral tissues and convey them to the central nervous system. The central terminals of these nociceptors innervate the substantia gelatinosa (lamina II) spinal dorsal horn, or the trigeminal nucleus of the brainstem in case of those nociceptors coming from the facial skin or the meninges.

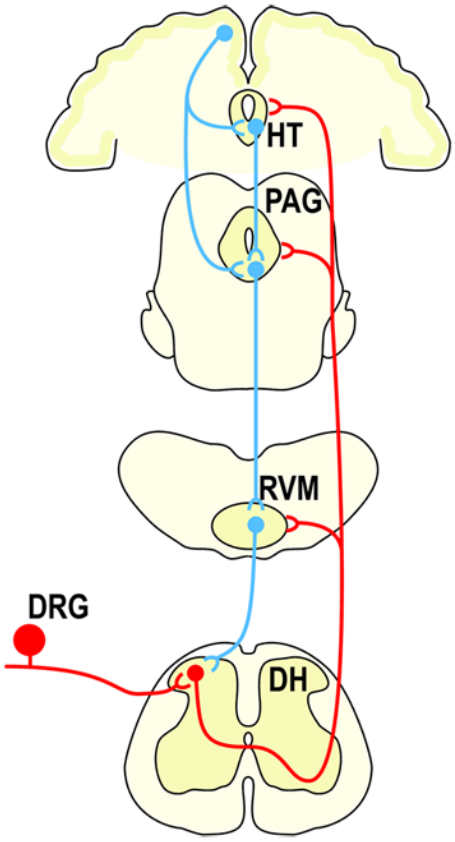

Figure 1. Schematic description of ascending pain pathways and descending anti-nociceptive fiber tracts.
Nociceptive ("painful") signals are conveyed by sensory fibers whose cell bodies reside in the dorsal root ganglia (DRGs). They reach the CNS at the level of the spinal dorsal horn (DH), where nociceptor terminals release glutamate to excite postsynaptic second order neurons. These central neurons transmit the nociceptive information via the brainstem and the midbrain to cortical areas, where the conscious sensation of pain arises. Descending antinociceptive pathways are controlled by cortical areas, which contact the hypothalamus (HT) and the periaqueductal grey (PAG). The PAG in turn controls the rostral ventromedial medulla (RVM), which constitutes the main origin of descending antinociceptive fibers innervating the spinal cord.

From there, signals are propagated through various relay stations in the brainstem, midbrain and thalamus to several cortical areas which give rise to the conscious sensation of pain. One site that has attracted particular attention in pain-related neuroplasticity is the spinal dorsal horn, which constitutes as the first site of synaptic integration in the pain pathway. Neurons

located in the spinal dorsal horn integrate primary afferent sensory signals of painful and non-painful modalities with input from descending fiber tracts, which can either inhibit or facilitate pain. Inhibitory interneurons have been attributed a critical role in this process already in the gate-control-theory of pain (figure 2, Melzack & Wall, 1965).

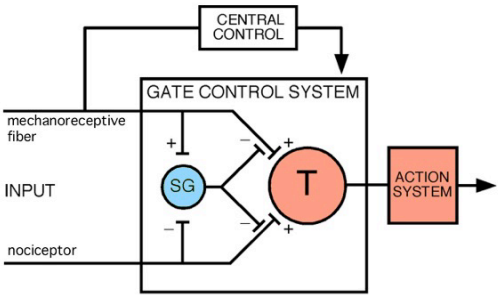

Figure 2. Gate-control theory of pain (Wall & Melzack, 1969).
Inhibitory neurons located in the substantia gelatinosa (SG) control the spinal pain gate. According to the original concept these neurons were controlled by non-nociceptive input from mechanosensitive fibers and by nociceptive input in opposite directions. Their activation would in turn control the spinal output system (T).

Although some of the synaptic connections proposed in the original scheme do apparently not exist, plenty of evidence indicates that compromising the function of inhibitory dorsal horn neurons induces symptoms reminiscent of chronic pain syndromes in humans. Animals develop an exaggerated sensitivity to painful stimuli (hyperalgesia), they respond with withdrawal responses upon exposure to stimuli, which are normally not felt as painful (allodynia), and they also show signs of spontaneous discomfort. Many lines of evidence indicate that typical causes of chronic pain such as inflammation or neuropathies compromise the function of inhibitory interneurons in the spinal dorsal horn through different mechanisms (for a review see Zeilhofer et al., 2012a). According to this concept, a facilitation of inhibitory neurotransmission should be a rational strategy for the treatment of many chronic pain states. Yet so far, none of the established analgesics acts through a facilitation of inhibitory neurotransmission. In the following text, we will review mechanisms of pain-related spinal disinhibition and evidence supporting the concept that novel subtype-selective benzodiazepine agonists would be suitable for the treatment of chronic pain syndromes. In the context of this review, we use the term "benzodiazepine" for all agonists at the benzodiazepine binding site of $GABA_A$Rs independent of their chemical structure. It should also be mentioned here that $GABA_A$Rs exist, which are resistant to modulation by classical benzodiazepines. These receptors contain $\alpha4$ or $\alpha6$ subunits instead of $\alpha1$, $\alpha2$, $\alpha3$, or $\alpha5$, or a $\gamma1$ or δ subunit instead of the $\gamma2$ subunit. These benzodiazepine-insensitive receptors are quite abundant in several brain regions (e.g. thalamus and cerebellum), but their expression in the spinal cord is very sparse.

Synaptic disinhibition in pathological pain

Fast synaptic inhibition in the spinal dorsal horn is mediated by GABA and glycine acting respectively at $GABA_AR$ and strychnine-sensitive GlyRs. Plenty of evidence indicates that blockade of spinal $GABA_ARs$ or GlyRs produces signs of allodynia and spontaneous pain (Beyer et al., 1985; Miraucourt et al., 2007; Roberts et al., 1986). More recent studies provided insights into the mechanism of this sensitization on the level of dorsal horn neuronal circuits. The most consistent observation in these studies was a strong increase in polysynaptic input onto lamina II neurons after application of the $GABA_AR$ antagonist bicuculline (Baba et al., 2003). A second finding was related to the synaptic input of lamina I projection neurons, which express the neurokinin 1 (NK1) receptor. These neurons serve an essential role in the relay of pathological pain, as their ablation strongly reduces hyperalgesia induced by inflammation and neuropathy (Nichols et al., 1999). Under normal conditions, these neurons receive sensory input almost exclusively from nociceptors (C and Aδ fibers). Blockade of $GABA_A$ and GlyRs however led to the *de novo* appearance polysynaptic responses from Aβ fibers (Torsney & MacDermott, 2006). These newly appearing polysynaptic connections likely underlie the allodynia seen *in vivo* after spinal application of bicuculline or strychnine. An increase in polysynaptic Aβ fiber input onto substantia gelatinosa (lamina II) may also occur as an endogenous process in chronic pain states (Baba et al., 1999).

Several groups have identified signaling pathways that reduce inhibitory synaptic transmission in inflammatory or neuropathic pain states (figure 3). A prostaglandin E2-mediated phosphorylation of superficial dorsal horn GlyRs renders these receptors less responsive to glycine (Ahmadi et al., 2002; Harvey et al., 2004; Reinold et al., 2005). Peripheral nerve damage leads to a down-regulation of the GABA synthesizing enzyme GAD65 in the spinal cord (Moore et al., 2002), and both inflammation and nerve injury cause an epigenetic down-regulation of the same enzyme in the brainstem (Zhang et al., 2011). A large number of neuromodulators interfere with the release of GABA and glycine from inhibitory dorsal horn neurons via activation of G protein coupled receptors and inhibition of Ca^{2+} channels (Zeilhofer et al., 2012b). An endocannabinoid and CB1 receptor-mediated inhibition of glycine and/or GABA release contributes to spinal sensitization evoked by extensive nociceptive input to the dorsal horn (Pernia-Andrade et al., 2009). Microglia activated in the dorsal horn in response to peripheral nerve damage down-regulates the

expression of the potassium and chloride co-exporter KCC2 in superficial dorsal horn neurons thereby shifting the reversal potential of GABA and glycine evoked chloride currents to more depolarized values. This shift renders glycinergic and GABAergic input less inhibitory (Coull et al., 2005; Coull et al., 2003; Keller et al., 2007), or, if the shift is sufficiently large, glycinergic and GABAergic input may even become excitatory and trigger action potentials in postsynaptic neurons (Coull et al., 2003).

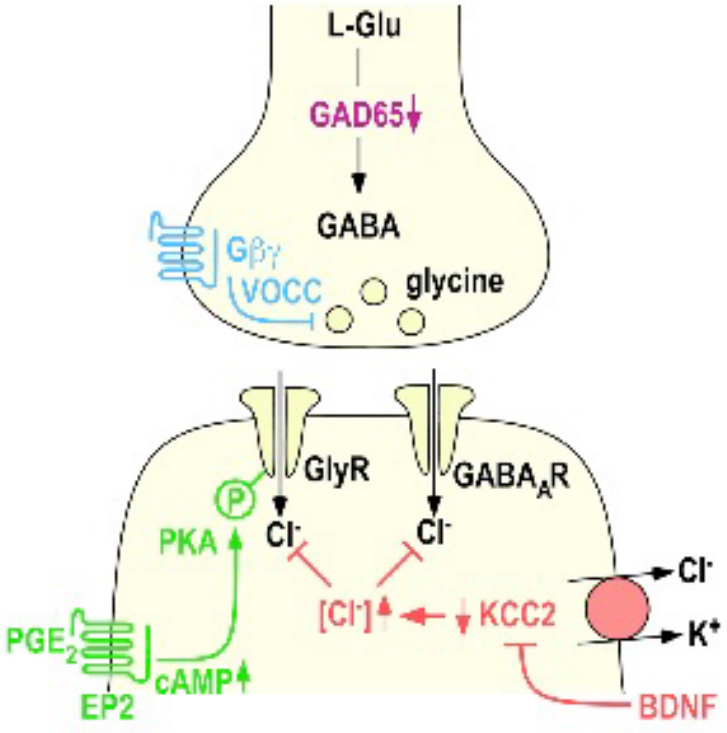

Figure 3. Four signaling pathways leading to spinal disinhibition in pathological pain states.
(1) Prostaglandin E2 (PGE_2) produced in the spinal cord in response to peripheral inflammation in creases cAMP production after activation EP2 receptors (EP2). The subsequent activation of protein kinase A (PKA) phosphorylates and inhibits GlyR of the superficial dorsal horn. (2) Peripheral nerve damage activates spinal microglia which releases brain-derived neurotrophic factor (BDNF). BDNF down-regulates the expression of the potassium/chloride exporter KCC2 leading to an increase in intracellular chloride ($[Cl^-]_i$). As a consequence GABAergic and glycinergic input becomes less inhibitory (or even excitatory). (3) Several neuromodulators including endocannabinoids reduce presynaptic GABA and glycine release rendering dorsal horn neurons more excitable. (4) Peripheral nerve damage leads to the down-regulation of the GABA synthesizing enzyme GAD65 and possible to reduced GABA content in inhibitory dorsal horn neurons.

Pharmacological enhancement of GABAergic synaptic transmission in the dorsal horn should be able to reverse pathological pain states that result from reduced presynaptic GABA release or from reduced responsiveness of postsynaptic $GABA_A$Rs. Some of the disinhibitory processes discussed above do specifically reduce glycinergic inhibition prompting the question whether a potentiation of GABAergic responses would be able to restore proper

inhibition in these cases. Many inhibitory dorsal horn neurons, co-release GABA and glycine from the same terminals and even from the same vesicles (Bohlhalter et al., 1994; Colin et al., 1998; Feng et al., 2005; Todd & Sullivan, 1990; Todd et al. 1996). In most dorsal horn neurons inhibitory postsynaptic responses are mediated by $GABA_AR$ and GlyRs (Baccei & Fitzgerald, 2004; Yoshimura & Nishi, 1995) and even in cells, in which no GABAergic component is visible under normal conditions, a GABAergic IPSC component can be revealed with benzodiazepines and neurosteroids (Keller et al., 2004; Keller et al., 2001). It is thus conceivable that pharmacological enhancement of GABAergic neurotransmission would also compensate for reduced glycinergic transmission.

The situation is more complex in those cases where disinhibition results from changes in the transmembrane chloride gradient. As long as the activation of $GABA_AR$ or GlyRs remains below the threshold of action potential activation, potentiation of $GABA_AR$ or GlyR may still remain inhibitory. However, as soon as the chloride equilibrium potential reaches the action potential threshold, potentiation of $GABA_AR$ or GlyR would increase the risk of paradoxical GABAergic and glycinergic excitation (Prescott et al., 2006). We discuss this issue below in the context of preclinical studies on subtype-selective benzodiazepines.

Spinal $GABA_AR$ subtypes mediating antihyperalgesia: evidence from genetically engineered mice

Analgesic or antihyperalgesic actions of benzodiazepines occur after local spinal injection suggesting that these effects are mediated by $GABA_ARs$ expressed in the spinal cord. To identify the $GABA_AR$ subtypes responsible for these antihyperalgesic effects, "knock-in" mice were investigated, in which the $\alpha1$, $\alpha2$, $\alpha3$, or $\alpha5$-$GABA_AR$ subunits had been rendered diazepam-insensitive through the introduction of a histamine to arginine (H/R) point mutation (Knabl et al., 2008; for information on the different point-mutated mouse strains see Rudolph & Möhler, 2004). In wild-type mice, intrathecal diazepam strongly reduced hyperalgesia in models of inflammatory or neuropathic pain, but had no effects on acute nociceptive pain. This "antihyperalgesic" activity was unchanged in mice, which carried the H/R point mutation in the $\alpha1$ subunit, but strongly reduced in mice, which carried the point mutation in the $\alpha2$ subunit. Mice with point-mutated $\alpha3$ or $\alpha5$ subunits showed reduced antihyperalgesic activity in some but not in all tests. The different subtypes of

benzodiazepine-sensitive $GABA_AR$s contribute to spinal antihyperalgesia with the rank order $\alpha2>\alpha3\geq\alpha5>>\alpha1$. The $GABA_AR$ subtype-dependence of spinal antihyperalgesia hence matched well with the expression of the different α subunits in the superficial dorsal horn (Bohlhalter et al., 1996; Lorenzo et al., 2014; Paul et al., 2012).

The lack of a contribution from $\alpha1$-$GABA_AR$s, which mediate the sedative effects of diazepam (Rudolph et al., 1999), demonstrates that antihyperalgesia by benzodiazepines can be studies in the absence of confounding sedation as long as $\alpha1$-$GABA_AR$s are not activated. Such experiments were performed in mice carrying H/R point mutations in the $\alpha2$, $\alpha3$, or $\alpha5$ subunits in addition to $\alpha1$ (Knabl et al., 2009). These experiments showed that antihyperalgesia could also be obtained after systemic diazepam (and in the absence of sedation) and that $\alpha2$ and $\alpha3$-$GABA_AR$ subtypes were the most relevant subtypes also for antihyperalgesia following systemic administration.

So far, $GABA_AR$ point-mutated mice have been used to assess the antihyperalgesic properties of benzodiazepines in three pain models, i.e. against zymosan A-induced inflammatory hyperalgesia, against neuropathic hyperalgesia induced by chronic constriction injury (CCI) of the sciatic nerve and in the formalin test. $GABA_AR$ subtypes mediating antihyperalgesia are thus clearly different from those mediating sedation (Rudolph et al., 1999), amnesia (Rudolph et al., 1999), and the rewarding properties of classical benzodiazepines (Tan et al., 2010). In the case of the $\alpha2$- and $\alpha3$-$GABA_AR$s, there is a clear overlap with the receptors mediating anxiolysis (Löw et al., 2000) and muscle relaxation (Crestani et al., 2001), and in case of $\alpha5$-$GABA_AR$ possibly also with those responsible for benzodiazepine-induced cognitive impairment (Dawson et al., 2006). For a comparison of the contribution of the different $GABA_AR$ subtypes to desired hyperalgesia and other effects see figure 4 E.

The antihyperalgesic efficacy of diazepam after systemic administration prompts two questions. How important are spinal versus supraspinal CNS areas for antihyperalgesia by systemic benzodiazepines? Do central effects such as a reversal of anxiety-induced hyperalgesia (Andre et al., 2005; Vidal & Jacob, 1982) indirectly contribute to the antihyperalgesia by systemic benzodiazepines? The latter question appears relevant in particular because $\alpha2$-$GABA_AR$s mediate not only antihyperalgesia but also anxiolysis (Löw et al., 2000). These questions were addressed with conditional $GABA_AR$ deficient mice (*hoxb8*-$\alpha2^{-/-}$ mice), which lack the $GABA_AR$ $\alpha2$ subunit specifically from the spinal cord and

dorsal root ganglia (DRGs) (up to about segment C4). In these experiments, a recently developed benzodiazepine site agonists (HZ166; Rivas et al., 2009) was employed which exerts antihyperalgesic actions similar to systemic diazepam but with reduced sedative and muscle relaxant properties (Di Lio et al., 2011). Antihyperalgesia was assessed as the change in heat and pin-prick induced withdrawal responses. These withdrawal responses are under strong control from descending pain modulating fiber tracts from various CNS areas (Carrasquillo & Gereau, 2007; Harris & Westbrook, 1995; Jasmin et al., 2003; Tatsuo et al., 1999) enabling the analysis of effects of descending (facilitating or inhibitory) pain modulation. Mice lacking the $GABA_AR$ α2 subunits specifically from the spinal cord showed virtually the same reduction in benzodiazepine-induced antihyperalgesia as global α2 (H/R) point-mutated mice confirming that the spinal cord was the most relevant site for the antihyperalgesic action of benzodiazepines also after systemic administration (Paul et al., 2014). This finding also largely ruled out secondary effects such as a reversal of anxiety-induced hyperalgesia. In this context it should also be added that the spinal cords of *hoxb8*-$\alpha2^{-/-}$ mice completely lacked α2-$GABA_ARs$ indicating that the spinal terminals of fibers descending from supraspinal CNS areas to the spinal cord do not express α2-$GABA_ARs$.

Mechanisms of spinal benzodiazepine-mediated antihyperalgesia

Immunohistochemistry studies have identified specific spinal distribution patterns of $GABA_AR$ subunits (Bohlhalter et al., 1996; Paul et al., 2012, figure 4 A - D). These receptors are expressed on intrinsic dorsal horn neurons and on the central terminals of primary sensory nociceptors. Spinal antihyperalgesia may therefore originate either from classical postsynaptic inhibition mediated by $GABA_ARs$ on intrinsic dorsal horn neurons or from $GABA_ARs$ on nociceptor terminals which mediate presynaptic inhibition through so called primary afferent depolarization. Both processes are illustrated in figure 5. The availability of a "floxed" α2-$GABA_AR$ allele for conditional gene deletion allowed experiments distinguishing between these two possibilities.

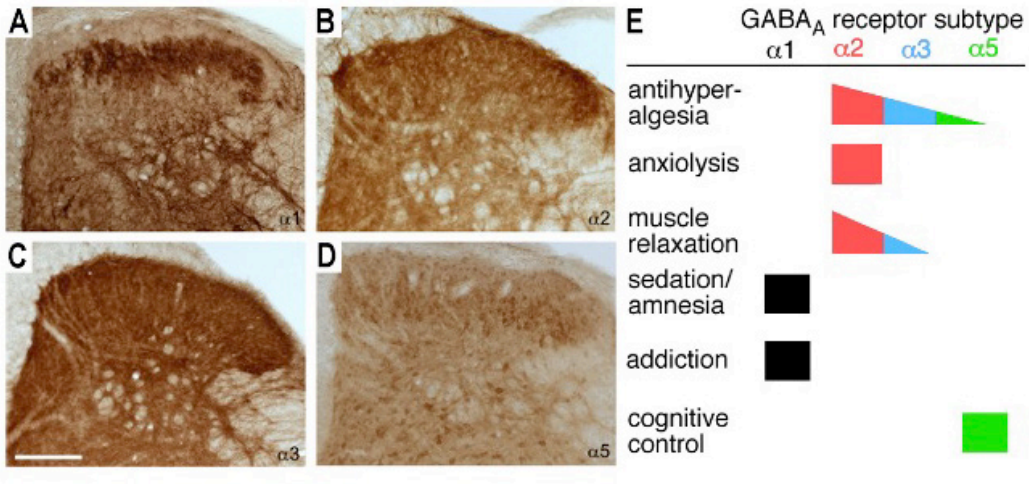

Figure 4. Distribution of α1, α2, α3, and α5-GABA$_A$R subunits in the lumbar spinal cord and contribution of the four subtypes of GABA$_A$Rs to antihyperalgesia.
(A - D) Immunocytochemical analysis of the expression of GABA$_A$R a subunits in the spinal dorsal horn of mice (reproduced from Paul et al., 2012). Scale bar, 100 µm. (E) Contribution of the different GABA$_A$R subtype to spinal antihyperalgesia and comparison with other behavioral effects of benzodiazepines (modified from Zeilhofer et al., 2009a).

Contribution of presynaptic inhibition and primary afferent depolarization

To assess the contribution of presynaptic inhibition, the α2-GABA$_A$R subunit was ablated specifically from nociceptor terminals using an sns::cre BAC transgenic mouse which expresses the cre recombinase under the transcriptional control of the *scn10* (Nav1.8) gene (Agarwal et al., 2004). In the case of an inflammatory pain model, the degree of antihyperalgesia by spinally applied diazepam in the nociceptor-specific α2-GABA$_A$R subunit deficient (sns-α2$^{-/-}$) mice fell between that measured in wild-type mice and global α2-GABA$_A$R point-mutated mice. The partial loss of diazepam-induced antihyperalgesia in the inflammatory model clearly indicated a contribution of presynaptic inhibition / primary afferent depolarization to antihyperalgesia by intrathecal diazepam. This was different in a neuropathy model in which all three genotypes responded with virtually identical antihyperalgesia (Witschi et al., 2011). Unaltered efficacy in the neuropathy model either indicates that antihyperalgesia was entirely due to postsynaptic inhibition of intrinsic dorsal horn neurons, or that antihyperalgesia occurred through inhibition of cre-negative (non-nociceptive) fibers. It has indeed been shown that inflammatory and neuropathic hyperalgesia depend on different classes of sensory fibers with Nav1.8 (sns) expressing sensory neurons being particularly important for inflammatory pain (Abrahamsen et al., 2008).

The results obtained in nociceptor-specific $\alpha2^{-/-}$ mice show that at least part of the antihyperalgesia originates from enhanced presynaptic inhibition and primary afferent depolarization. The remaining α2-$GABA_AR$-mediated component may result either from primary afferent depolarization of non-nociceptive fibers or from postsynaptic inhibition of intrinsic dorsal horn neurons. In both cases, the complete loss of α2-$GABA_AR$-mediated antihyperalgesia in *hoxb8*-$\alpha2^{-/-}$ mice unequivocally demonstrates that the major component of benzodiazepine-evoked antihyperalgesia is of spinal origin (Paul et al., 2014). This has not yet been formally proven for the α3- and α5-$GABA_AR$-mediated components. However, both subunits are also enriched in the dorsal horn and their antihyperalgesic actions may thus also come from the spinal cord.

Mechanisms of presynaptic inhibition

It is well established that the spinal terminals of primary sensory neurons carry functional benzodiazepine-sensitive $GABA_ARs$. Activation of these presynaptic $GABA_ARs$ causes depolarization of sensory neurons rather than hyperpolarization because primary afferent sensory neurons lack an efficient chloride export mechanism (Kanaka et al., 2001; Price et al., 2006). As a consequence, the intracellular chloride concentration in these neurons renders the chloride equilibrium potential more positive than the resting membrane potential. This depolarization is however still inhibitory probably because it leads to a voltage-dependent inactivation of Na^+ and Ca^{2+} channels in the axon and the axon terminal, respectively, and subsequently reduces transmitter release (Kullmann et al., 2005). This presynaptic inhibition can occur through axo-axonic synapses. Their existence is firmly established for non-nociceptive primary sensory fibers (Aβ and low threshold Aδ fibers; Ribeiro-da-Silva, 1995). Axo-axonic contacts have also been found in nociceptor terminals, but less frequently than in terminals of non-nociceptive fibers (Alvarez et al., 1993; Ribeiro-Da-Silva et al., 1986; Ribeiro-da-Silva et al., 1989). Two recent studies disagree on the presence of gephyrin clusters on nociceptor terminals (Lorenzo et al., 2014; Paul et al., 2012). Because gephyrin is required for postsynaptic clustering of inhibitory neurotransmitter receptors in central neurons, the presence or absence of gephyrin clusters from sensory fiber terminals may be taken as an argument in favor or against the presence of axo-axonic synapses between GABAergic interneurons and nociceptor terminals. Physiological studies have established that primary afferent depolarization and presynaptic inhibition exist also in nociceptors (Lin et al., 1999; Lin et al., 2000; Witschi et al., 2011). Nociceptor terminals lacking GABAergic

axo-axonic synapses may undergo to presynaptic inhibition through $GABA_AR$ via "spill-over" of GABA from neighboring synapses and so called volume transmission (figure 5; Rudomin & Schmidt, 1999, for a more recent review see also Zeilhofer et al., 2012b).

Antihyperalgesic action of benzodiazepines with improved subtype-specificity: preclinical studies

A number of benzodiazepines with reduced activity at α1-$GABA_ARs$ have been developed in the last two decades mainly in the quest for non-sedating anxiolytics (for a comprehensive list see (Rudolph & Knoflach, 2011). Because benzodiazepine-mediated anxiolysis and antihyperalgesia share a similar dependence on $GABA_AR$ subtypes, some of these compounds were also tested in pain studies (table 1). Some of these compounds are sometimes referred to as "α2/3 selective" (e.g. Hofmann et al., 2012), this is however incorrect as all of them are also agonists at α5-$GABA_ARs$. This should not be forgotten when undesired effects of these compounds are discussed. NS11394, which has very low activity at α1-$GABA_ARs$ (<10% relative to diazepam) and good partial agonistic activity at α2-, α3-, and α5-$GABA_ARs$ (26 – 78%, relative to diazepam) (Mirza et al., 2008), exhibited antinociceptive activity at non-sedating doses in several rodent pain models (Hofmann et al., 2012; Munro et al., 2008). The analgesic effect in the formalin test was blocked by the benzodiazepine site antagonist flumazenil (Munro et al., 2008) confirming that it occurred through $GABA_ARs$. L-838,417, which completely lacks intrinsic activity at α1-$GABA_ARs$ and possesses partial agonistic activity at α2-, α3-, and α5-$GABA_ARs$ (15 – 32%, relative to diazepam; McKernan et al., 2000), was tested in several rodent pain models where it was not sedative but active against inflammatory and neuropathic hyperalgesia (Knabl et al., 2008; Nickolls et al., 2011) and formalin-induced nociception (Hofmann et al., 2012).

In addition, L-838,417 was active against hyperalgesia evoked by skin incision, a model of postoperative hyperalgesia (Reichl et al., 2012) and against capsaicin-induced central pain sensitization (Hansen et al., 2012). Other benzodiazepines with low sedative propensities include HZ166 (Rivas et al., 2009) and TPA023 (Atack et al., 2006). HZ166, which has high intrinsic activity, was antihyperalgesic in inflammatory and neuropathic mouse models (Di Lio et al., 2011), while TPA023, which is a low intrinsic activity partial agonist, showed comparatively weak anti-allodynic or antihyperalgesic effects (Munro et al, 2011; Nickolls et

al., 2011). Other compounds, which have higher intrinsic activities at α1-$GABA_A$Rs than at α2-, α3-, and α5-$GABA_A$Rs, did not exhibit antihyperalgesic activity at non-sedative doses. These results are fully consistent with the results obtained from H/R point-mutated mice.

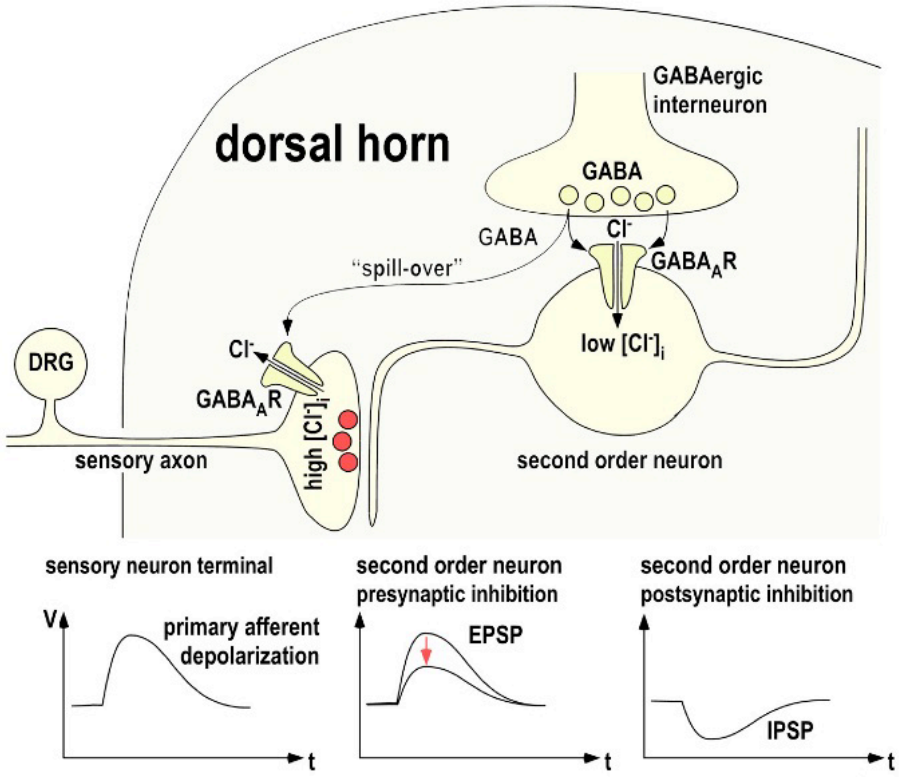

Figure 5. Mechanisms of the GABAergic control of spinal pain transmission.
(A) Anatomical arrangement of pre- and postsynaptic inhibition. (B) Activation of $GABA_A$Rs on nociceptor terminals causes chloride efflux and primary afferent depolarization (left panel). This primary afferent depolarization causes a presynaptic inhibition of synaptic glutamate release from nociceptor terminals (middle panel). Activation of $GABA_A$Rs on second order neurons (intrinsic) dorsal horn neurons activates chloride influx and causes classical postsynaptic inhibition through hyperpolarization and dendritic shunting (right panel).

Novel benzodiazepines for treatment of chronic pain would not only have to have good analgesic or antihyperalgesic efficacy and low or at least weak sedative properties, but would also have to avoid other typical side effects of benzodiazepines such as addiction and tolerance development (i.e. a loss of activity during prolonged treatment).

Addiction

Reinforcing (or addictive) properties were absent or reduced in the case of partial agonists which lack efficacy at α1-$GABA_A$Rs, including L-838,417 (Rowlett et al., 2005) and

TPA023 (Abe et al., 2009). Other partial agonists with higher efficacy at α1-$GABA_A$Rs such as TPA123 caused a withdrawal syndrome following upon cessation of self-administration, suggesting that the lack of agonistic activity at α1-$GABA_A$Rs was the relevant parameter rather than a general reduction in agonistic activity (Ator et al., 2010). These results are consistent with studies in α1-$GABA_A$R point-mutated mice, in which no reinforcement was seen with the full agonist midazolam (Tan et al., 2010).

Tolerance development against antihyperalgesia

Analgesia by opioids shows a high liability to tolerance development and the same applies to many of the actions of classical benzodiazepines including antihyperalgesia in rats (Witschi & Zeilhofer, unpublished) and mice (Ralvenius et al., 2013). Whether tolerance against antihyperalgesia occurs also with more selective or non-sedative benzodiazepines has been studied for three compounds. L-838,417 was devoid of tolerance developments against antihyperalgesic / analgesic actions during chronic nine day treatment, while an equally effective dose of morphine completely lost its antihyperalgesic activity during the same time period (Knabl et al., 2008). Tolerance development was also not observed against antihyperalgesia by HZ166 (Di Lio et al., 2011), whereas NS11394 lost at least part of its analgesic activity against formalin-induced pain during chronic eight-day treatment (Hofmann et al., 2012). It is at present unknown whether reduced tolerance liability of non-sedative or more selective benzodiazepines is due to their reduced intrinsic activity (partial agonism), to improved subtype-selectivity, or to pharmacokinetic differences. Mice carrying different combinations of point-mutated $GABA_A$R α subunits should offer a tool to address these possibilities.

Clinical studies on antihyperalgesia by benzodiazepines

The preclinical studies discussed above performed in mice resistant to the sedative effects of benzodiazepines demonstrate that classical benzodiazepines do in principle exert profound antihyperalgesic actions but only at doses, which normally induce strong sedation. Less sedating benzodiazepines exhibited antihyperalgesic efficacy at non-sedating doses also in wild-type mice.

In human patients, classical non-selective benzodiazepines do not exert relevant analgesic (or antihyperalgesic) actions at clinically used doses. The few publications that found positive evidence for efficacy in pain patients were pilot studies or open trials (e.g. Fishbain et al., 2000; Harkins et al., 1991), which provide only limited evidence. Possible explanations for this lack of analgesic or antihyperalgesic efficacy in humans include (1) species differences (rodents versus humans), (2) biological differences between preclinical models of pain and pain in human patients, and (3) dose limiting sedation after systemic administration of classical benzodiazepines. The authors of this review favor the latter possibility for several reasons.

A recent study investigated possible analgesic or antihyperalgesic effects of two classical benzodiazepines (clobazam and clonazepam) in a battery of pain tests in human volunteers (Vuilleumier et al., 2013). This study found of a (small) antihyperalgesic effect in several parameters including in the size of the hyperalgesic area induced by intracutaneous capsaicin injection and in several muscle pain-related read-outs. Non-selective full agonists such as diazepam, clonazepam, midazolam typically induce strong sedation or sleep already at receptor occupancies between 15 and 30% (Fujita et al., 1999; Malizia et al., 1995; Pauli et al., 1991; Shinotoh et al., 1989). Compounds with a better $\alpha2/\alpha1$-selectivity ratio should permit higher levels of receptor occupancy and hence higher $\alpha2$-$GABA_AR$ activation before reaching dose-limiting sedation.

The data available from the few benzodiazepines with improved selectivity profile that went into clinical trials provide some insight as to what degree of selectivity would be needed to avoid sedation in humans. One such study investigated MK-0343 (also known as MRK-409), which has very weak agonistic activity at $\alpha1$-$GABA_ARs$ (18% relative to the full agonist chlordiazepoxide; Atack et al., 2011). This compound was sedative in humans although previous preclinical tests in rodents had not shown any evidence for sedative properties (Atack et al., 2011; de Haas et al., 2008). A related compound (TPA023B) fully devoid of agonistic activity at $\alpha1$-$GABA_ARs$ did not produce sedation in man (Atack et al., 2011). These results suggest that the human brain is more susceptible to sedative actions of benzodiazepines than that of mice and rats (or, alternatively one might argue that our tests to assess sedation on rodents are less sensitive than those in man). In both cases the available data indicate that sedation in humans can be avoided with compounds fully devoid of intrinsic activity at $\alpha1$-$GABA_ARs$. Concert Pharmaceuticals is currently performing a phase 1

clinical trial on a deuterated version of L-838,417 (now called CTP-354), which has more favorable pharmacokinetics in humans than L-838,417 (http://www.concertpharma.com/CTP354Phase1Initiation.htm). It would be very informative to see whether CTP-354 or TPA023B, which has an intrinsic activity at α2-$GABA_A$Rs even higher than that of L-838,417 and no agonistic activity at α1-$GABA_A$Rs, possess antihyperalgesic or analgesic activity in human volunteers or pain patients.

Open questions

Which $GABA_A$R subtypes should be targeted for optimal analgesia with minimal side-effects?

Work in the $GABA_A$R H/R point-mutated mice suggests that α2-, α3-, and α5-$GABA_A$Rs contribute to spinal benzodiazepine antihyperalgesia. It is however not clear whether only one subtype (i.e. α2-$GABA_A$Rs) should be targeted for optimal antihyperalgsia or whether simultaneous activity at more than one subunit would be advantageous. In the absence of fully selective subtype-specific drugs, investigations on mice carrying more than one point mutated $GABA_A$R subtype should be informative. When taking undesired effects into consideration, one would probably try to avoid positive allosteric modulation of α5-$GABA_A$Rs as this modulation might confer cognitive impairment. Another reason to avoid activity at α5-$GABA_A$Rs comes from a recent report suggesting that inverse agonistic activity at α5-$GABA_A$Rs could be analgesic (Munro et al., 2011). So far, one would not expect undesired effects from activity at α3-$GABA_A$Rs, however it is at present unknown if development of tolerance against the α2-mediated antihyperalgesic activity can be avoided by sparing activity at other $GABA_A$R subtypes. This is again a question, which is potentially accessible to studies examining mutant mice carrying combinations of H/R point-mutated $GABA_A$R subunits.

The genetic approaches described in this review are very well suited for experiments addressing the function of precisely defined $GABA_A$R subtypes. It is however not clear whether structural differences in the benzodiazepine binding site of different $GABA_A$R subtypes are large enough to permit development of subtype-selective benzodiazepines for each subtype or subtype combination. In particular differences between α2 and α3 subunits

might be too small to permit subtype specificity. Searching for modulatory sites at $GABA_ARs$ different from the classical benzodiazepine binding site might offer an alternative very intriguing, yet so far largely unexplored, opportunity.

Mixed $GABA_ARs$ with more than one type of α subunit

One potential limitation of the H/R point mutation approach is the behavior of $GABA_ARs$ with more than one type of α subunit. More than 25% of α1 containing $GABA_ARs$ contain a second α subunit different from α1 (mainly α2 or α3), and the majority of α2 and α3 $GABA_ARs$ are mixed receptors (Benke et al., 2004). Because only one of the two α subunits can interact with the γ subunit to form the high affinity benzodiazepine binding site, the responses of these mixed receptors to fully subtype selective compounds will be determined by the type of α subunit which associates with the γ subunit (Minier & Sigel, 2004). This association may occur randomly but biochemical data suggest a certain rank order of the α subunits for interaction with the γ2 subunit (Balic et al., 2009). Moreover, biochemical experiments suggest that the H/R point mutation in the α subunit not only abolishes modulation by diazepam but also impairs the interaction with the γ subunit. This may eventually lead to false negative results in experiments with H/R point-mutated mice and may contribute to some of the discrepancies between predictions made from the H/R point-mutated mice and results obtained with subtype-selective agents (Sigel & Steinmann, 2012; Skolnick, 2012). If differences in the prevalence of the different types of mixed $GABA_ARs$ exist between mice and humans, these differences may also explain some of the discrepant results obtained in rodent models and in clinical studies. Experiments comparing the phenotypes of single point-mutated mice and of triple point-mutated mice in which only a single subtype remains benzodiazepine-sensitive may provide additional insights also here.

Conclusions

There is compelling evidence from preclinical studies in rodents to support that non-sedative benzodiazepines with improved subtype specificity exert antihyperalgesic effects. Available clinical data are consistent with this view. Current knowledge suggests that robust antihyperalgesic activity with low sedative properties requires a high intrinsic activity at α2-$GABA_ARs$ (or possibly also at α3-/α5-$GABA_ARs$) and very low or little activity at α1-

$GABA_ARs$. The optimal profile of such a drug in terms of $GABA_AR$ subtype specificity is however still not known. On-going preclinical studies and clinical trials will hopefully soon provide additional insights.

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Conflict of interest statement

The authors declare that they have no conflict of interest

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2 AIMS

As described above, a dysfunction of the inhibitory system on a spinal level can lead to hyperalgesia and allodynia, which are hallmarks of chronic pain states. Potentiating the spinal GABAergic inhibition through intrathecal benzodiazepine injection has been shown to compensate for this disinhibition (Knabl et al., 2008). Studies in mice carrying point mutations in single $GABA_A$ receptors have provided first insights into the contribution of the different $GABA_A$ receptor subtypes. However, they did not provide a comprehensive analysis of desired and undesired effects expected from subtype-selective GABAergic drugs.

2.1 The first project of the present thesis was designed to address the following specific questions:

(1) what is the distribution of $GABA_A$ receptor subtypes (not subunits) in the spinal cord?

(2) which $GABA_A$ receptor subtype should be targeted to achieve maximal antihyperalgesia with minimal side-effects?

(3) why do classical benzodiazepines lack clinically relevant analgesic efficacy?

2.2 The second project of this thesis was designed to help translating the results of rodent studies on antihyperalgesic properties of $GABA_A$ receptor subtypes to clinical application.

None of the currently available subtype-selective $GABA_A$ receptor modulators developed by pharmaceutical industry in recent years is currently available for proof-of-concept studies in man. I therefore evaluated alternative approaches. N-desmethyl clobazam (NDMC) is a naturally occurring metabolite of the clinically used benzodiazepine clobazam (CBZ). Previous studies have suggested that NDMC might exhibit an improved $\alpha2/\alpha1$ $GABA_A$ receptor activity ratio.

The second part of my thesis was therefore designed to address the following specific aims:

(1) how does the in vitro pharmacological profile of NDMC compare to those of the parent compound CBZ and the prototypical benzodiazepine diazepam?

(2) what are the dose-dependencies of NDMC-evoked antihyperalgesia and sedation in comparison to CBZ?

(3) do side-effects different from sedation occur with NDMC at antihyperalgesic doses?

EXPERIMENTAL SECTION

ANALGESIA AND UNWANTED BENZODIAZEPINE EFFECTS IN POINT-MUTATED MICE EXPRESSING ONLY ONE BENZODIAZEPINE-SENSITIVE $GABA_A$ RECEPTOR SUBTYPE*

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Abstract

Agonists at the benzodiazepine-binding site of $GABA_A$ receptors (BDZs) enhance synaptic inhibition through four subtypes (α1, α2, α3, α5) of $GABA_A$ receptors ($GABA_ARs$). When applied to the spinal cord, they alleviate pathological pain, but insufficient efficacy after systemic administration and undesired effects preclude their use in routine pain therapy. Previous work suggested that subtype-selective drugs might allow separating desired antihyperalgesia from unwanted effects, but the lack of selective agents has hitherto prevented systematic analyses. Using four lines of triple $GABA_AR$ point-mutated mice, which expressed only one benzodiazepine-sensitive $GABA_AR$ subtype at a time, we found that strong antihyperalgesia and reduced side-effects (i.e. no sedation, motor impairment, and tolerance development) are best achieved when only α2$GABA_ARs$ are targeted. Additional pharmacokinetic/pharmacodynamic analyses in these mice explain why clinically relevant antihyperalgesia cannot be achieved with non-selective BDZs. These findings should strongly foster the development of innovative subtype-selective BDZs for novel indications including chronic pain.

Introduction

Chronic pain is a severe medical condition affecting hundreds of millions of patients world-wide. It is widely accepted that diminished inhibition in pain processing circuits of the spinal dorsal horn is a major contributor to different chronic pain forms (Zeilhofer et al., 2012; De Koninck, 2007; Kuner, 2010; Sandkühler, 2009). We have previously demonstrated that local spinal application of BDZ site ligands that positively modulate $GABA_AR$ function alleviates neuropathic and inflammatory pain in rodents (Knabl et al., 2008). Translation of these results to routine systemic pain treatment however requires a separation of desired antihyperalgesia from unwanted side-effects. This separation appears potentially feasible based on the existence of different $GABA_AR$ subtypes.

Most $GABA_ARs$ in the brain and spinal cord are heteropentameric ion channels composed of two α, two β and one γ2 subunit (Sigel et al., 2006). The high affinity binding site for BDZs is formed by an interface between one α subunit and the γ2 subunit. High affinity binding of BDZs at this site requires presence of a histidine residue at a conserved site in the N-terminal domain of the α subunit. This conserved histidine is present in α1, α2, α3, or α5 subunits, but not in α4 and α6 subunits (Wieland et al., 1992). Mutation of the histidine residue into an arginine dramatically reduces the affinity of $GABA_ARs$ to BDZs without changing their responses to GABA (Benson et al., 1998). The generation of histidine to arginine (H → R) point-mutated mice for each of the four BDZ-sensitive $GABA_AR$ subunits has allowed attributing the different *in vivo* effects of BDZs to defined $GABA_AR$ subtypes (Möhler et al., 2005). Most importantly, it was shown that the sedative effects of BDZs were strongly reduced in mice carrying the H → R point mutation in their α1 subunits (McKernan et al., 2000; Rudolph et al., 1999), while point mutating the α2 subunits led to a loss in the anxiolytic effects of BDZs (Löw et al., 2000). Using this approach, we could demonstrate that mice whose $\alpha2GABA_ARs$ had been rendered BDZ-insensitive show drastically reduced antihyperalgesic responses to spinal diazepam (DZP) (Knabl et al., 2008). A general consensus on the question, which $GABA_AR$ subtype should best be targeted to achieve maximal antihyperalgesic responses and to best avoid undesired effects has not been reached. A similarly open question is why classical BDZs are largely devoid of analgesic actions in patients.

Highly selective tool compounds that would allow addressing these questions pharmacologically are still lacking (Rudolph & Knoflach, 2011). For this reason, we have

generated triple $GABA_AR$ point-mutated mice, in which only a single $GABA_AR$ subtype remains BDZ-sensitive. We designate these mice hereafter as HRRR, short for α1H;α2R;α3R;α5R (i.e. a mouse in which only α1$GABA_ARs$ are left BDZ-sensitive) and, accordingly, as RHRR, RRHR, RRRH for mouse lines, in which either only α2, α3, or α5 subunits remain BDZ-sensitive. In these mice, the action of the classical normally non-selective BDZ agonist DZP should mimic the action of fully subtype-selective compounds. Such a genetic approach also avoids possible confounding factors of subtype-selective compounds such as pharmacokinetic differences and unknown specificity profiles of drug metabolites. With the use of triple $GABA_AR$ point-mutated mice, we were not only able to perform a systematic analysis on the $GABA_AR$ subtypes best targeted for optimal antihyperalgesia and least-pronounced side-effects but we also provide an explanation why classical (non-selective) BDZs are largely devoid of clinically relevant analgesic properties.

Results

Spinal distribution of subtype-specific BDZ binding sites

In the first series of experiments, we characterized the distribution pattern of the four subtypes of BDZ-sensitive $GABA_ARs$ ("BDZ receptors") in the spinal dorsal horn of triple point-mutated mice. Using immunocytochemistry, we verified that neither the regional distribution nor the expression levels of α1, α2, α3, and α5 subunits and of the γ2 subunit differed between wild-type (wt) mice and the four strains of triple point-mutated mice (Fig. 1 A,B). Similarly, the total number of BDZ binding sites (wt receptors plus H → R point-mutated receptors) quantified through [^{3}H]Ro15-4513 autoradiography was unchanged (Fig. 1 C). Spinal autoradiography with [^{3}H]flumazenil which binds with high affinity only to non-point-mutated $GABA_ARs$ allowed a quantitative analysis of each of the four subtypes of BDZ binding sites in isolation. In wt mice, [^{3}H]flumazenil exhibited specific binding throughout the spinal grey matter with enrichment in the dorsal horn and around the central canal. The density of binding sites in the dorsal horn was highest for the α3$GABA_AR$ subtype, followed by α2 and α1, and lowest for the α5 subtype (Fig. 1 D). The α1 subtype was concentrated around the central canal, α2 was most abundant in the superficial dorsal horn, where nociceptive fibers terminate, α3 was found throughout the dorsal horn and around the central canal, while α5 was generally weak. The distribution of subtype-specific [^{3}H]flumazenil

binding in the four strains of $GABA_AR$ triple point-mutated mice matched the distribution of the subunits determined with immunocytochemistry on a gross scale.

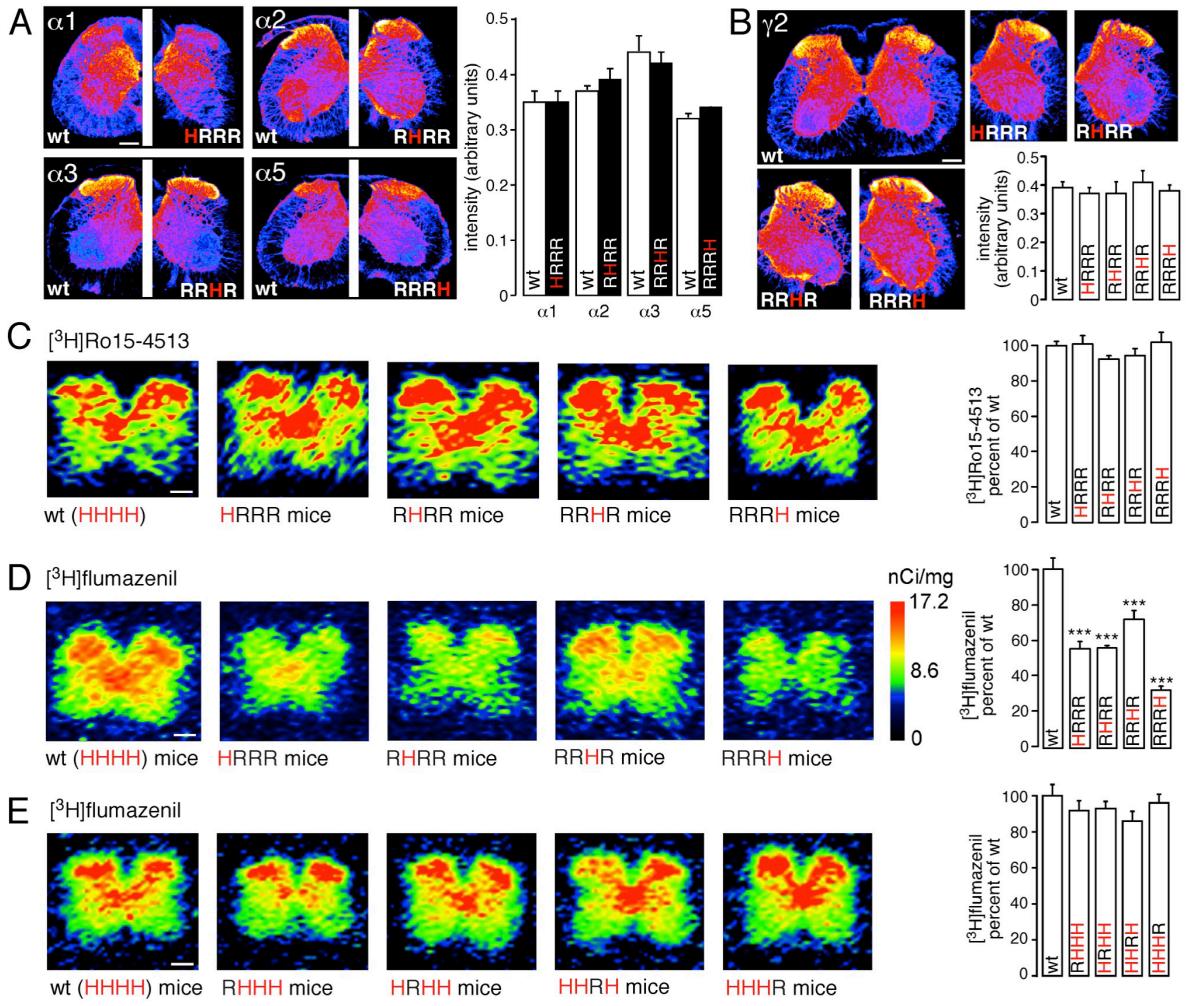

Figure 1. Distribution of the four subtypes of BDZ-sensitive $GABA_ARs$ in the spinal cord.
(**A,B**) Immunohistochemistry. (**A**) Localization of $GABA_AR$ α subunits in spinal cords of wt mice and of the four types of $GABA_AR$ triple point-mutated mice. Pseudocolor images display receptor density (lowest to highest density, blue to yellow). Right: quantitative comparison of expression levels in wt and triple point-mutated mice. Two-way ANOVA for interaction (α subunit x genotype): $F(7,42)=0.53$, $P=0.68$. n=6 mice (3 sections each) for all groups, except RRHR, where n=7 mice. (**B**) Same as (A) but for γ2 subunits. ANOVA followed by Dunnett's *post hoc* test. $F(4,15)=0.32$; $P\geq0.94$. n=4 mice (3 sections each) for all genotypes. (**C,D**) Autoradiography. (**C**) [^{3}H]Ro15-4513 binding sites indicate the distribution of all α/γ $GABA_ARs$ independent of their BDZ-sensitivity in wt mice and the four types of $GABA_AR$ triple point-mutated mice. H and R indicate homozygous wt and point-mutated alleles (for α1/α2/α3/α5 subunits), respectively. Right: quantification of [^{3}H]Ro15-4513 binding sites in the dorsal horn. ANOVA followed by Dunnett's *post hoc* test with wt as control. $F(4,10)=2.89$, $P\geq0.18$. n=3, for all genotypes (> 30 sections each). (**D**) Same as (C) but distribution of BDZ-sensitive $GABA_ARs$ labeled with [^{3}H]flumazenil. ANOVA followed by Dunnett's *post hoc* test with wt as control. $F(4,55)=40.1$, ***; $P<0.001$. n=11, 12, 12, 12, and 14 mice (> 10 sections each), for wt(HHHH), HRRR, RHRR, RRHR, and RRRH mice, respectively. (**E**) Same as (D) but wt and $GABA_AR$ single point-mutated mice. $F(4,42)=0.441$; $P>0.66$. n=11, 9, 9, 9, and 9 mice, for wt(HHHH), RHHH, HRHH, HHRH, and HHHR mice, respectively. All scale bars, 200 µm. All quantitative data mean ± SEM.

However, the sum of the binding sites in the four strains of triple point-mutated mice was about twice as high as the total number of binding sites in wt mice. The results from the immunocytochemistry and [^{3}H]Ro15-4513 autoradiography largely rule out changes in protein expression as the underlying cause. Instead, the [^{3}H]flumazenil data are consistent with a high prevalence of $GABA_ARs$ containing two different α subunits (Araujo et al., 1996; Benke et al., 2004) and with a model of the $GABA_AR$ assembly in which non-point-mutated α subunits have a higher probability for interaction with the γ2 subunit than H → R point-mutated subunits (Balic et al., 2009). This conclusion is further supported by the only marginal reduction of BDZ binding observed in spinal cords of the four single point-mutated mouse lines (Fig. 1 E).

Antihyperalgesia by single BDZ-sensitive $GABA_ARs$ subtypes

We next used the triple point-mutated mice to predict pharmacological actions of subtype-selective BDZ site agonists and treated them with DZP, a classical non-selective BDZ, whose agonistic activity was restricted to a single $GABA_AR$ subtype in triple point-mutated mice. We first focused on the antihyperalgesic potential of such an approach (i.e. the potential to reduce neuropathy-induced hyperalgesia) and tested DZP in mice subjected to the chronic constriction injury (CCI; Bennet & Xie, 1988) model. Baseline mechanical and heat sensitivity were statistically indistinguishable in all strains of mice analyzed and all strains of mice developed similar hyperalgesia after CCI (Tab. 1). All subsequent experiments with DZP were done on an α1R point-mutated background to avoid DZP-induced sedation, which is a potential confounding factor in behavioral pain tests. We first tested different doses of systemic (per os, p.o.) DZP in α1 point-mutated RHHH mice (Fig. 2 A,B). Based on these results, we chose a dose of 10 mg/kg body weight, which is close to the ED_{70} for subsequent experiments. We then asked which $GABA_AR$ subtype exerts the strongest antihyperalgesic action and tested the effects of DZP on mechanical (von Frey filament test) and heat (Hargreaves test) hyperalgesia in the four strains of triple $GABA_AR$ point-mutated mice (Fig. 2 C-E). For both stimuli, the strongest effect was achieved in RHRR mice in which only α2$GABA_ARs$ were BDZ-sensitive. Exclusive targeting of α3 and α5$GABA_ARs$ (in RRHR and RRRH mice) also elicited antihyperalgesia but to a smaller extent.

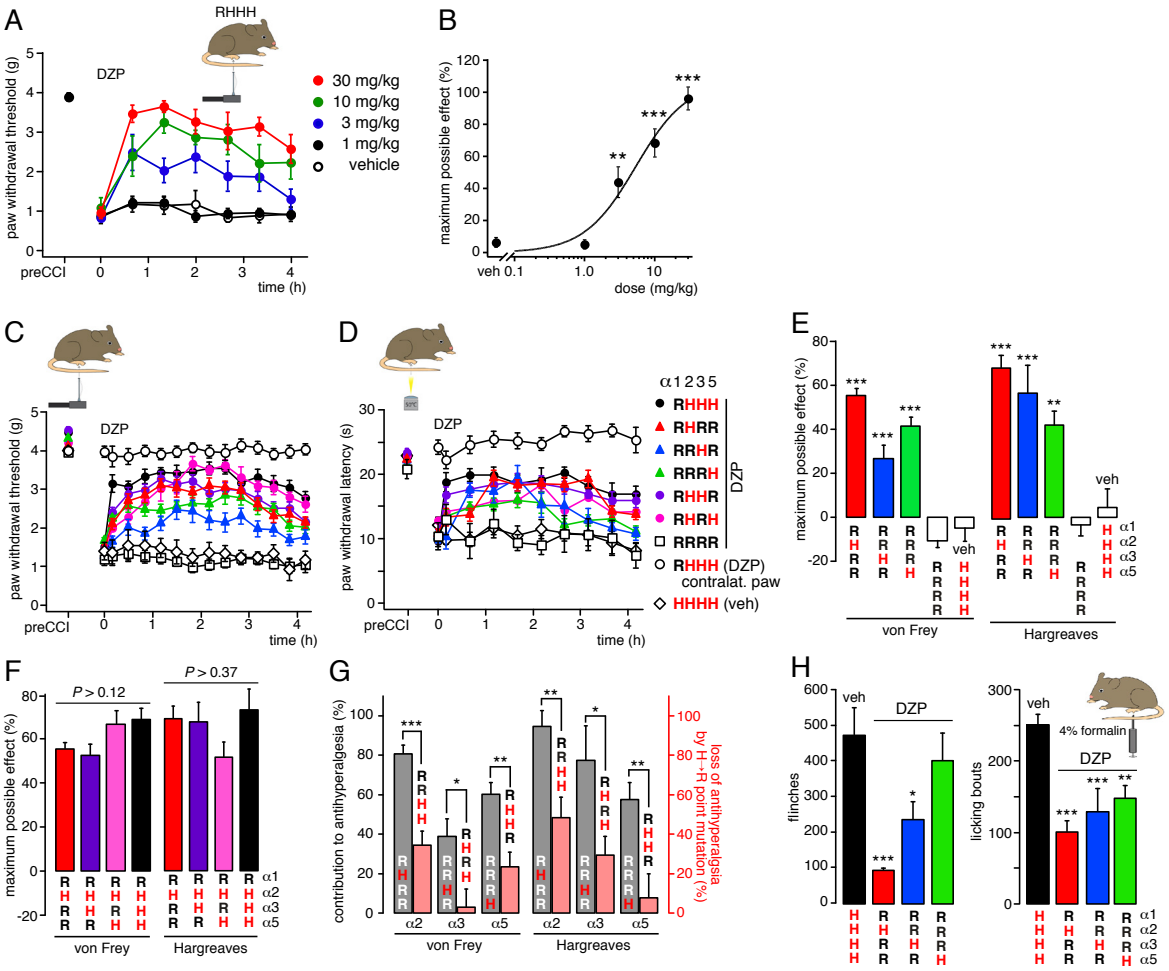

Figure 2. Antihyperalgesia by per oral DZP in GABA$_A$R point-mutated mice.
(A) Reversal of mechanical hyperalgesia by DZP in α1 point-mutated (RHHH) mice assessed with von Frey filaments 7 days after CCI surgery. (**B**) Dose-response relationship. Data were fitted to Hill's equation. ***, P<0.001; **, P<0.01 versus vehicle (ANOVA F(4,28)=29.0 followed by Dunnett's *post hoc* test. n=7, 7, 6, 7, and 6 mice, for vehicle and 1, 10, and 30 mg/kg DZP, respectively). (**C,D**) Reversal of mechanical (**C**) and heat hyperalgesia (**D**) by DZP (10 mg/kg) one week after CCI surgery. All mice except wt carried the H→R point-mutation in α1GABA$_A$Rs to avoid sedation. (**E**) Comparison of antihyperalgesia (determined from 70 to 130 min after DZP administration) in GABA$_A$R triple point-mutated mice. ***, P<0.001; **, P<0.01; versus vehicle-treated wt mice (ANOVA followed by Dunnett's *post hoc* test, F(4,54)=36.2 and F(4,53)=15.1 for von Frey and Hargreaves tests, respectively). No antihyperalgesia by DZP occurred in quadruple GABA$_A$R point-mutated mice (P=0.85 and 0.94). (**F**) Comparison of antihyperalgesia by DZP in RHRR mice with that of mice carrying in addition BDZ-sensitive α3 and/or α5GABA$_A$Rs. No significant differences were observed (ANOVA followed by Dunnett's *post hoc* test, von Frey: F(3,57)=2.96; P≥0.12. Hargreaves: F(3,44)=0.98; P≥0.37). (**G**) Antihyperalgesia in triple GABA$_A$R point-mutated mice with only one DZP-sensitive GABA$_A$R subtype (grey columns) compared to antihyperalgesia lost through H→R point mutation of the same subunit (light red columns). Antihyperalgesia in triple point-mutated mice is expressed as percentage of antihyperalgesia in RHHH mice. Loss of antihyperalgesia was calculated as the reduction in antihyperalgesia relative to RHHH mice. ***, P<0.001; **, P<0.01; *, P<0.05 (unpaired t-tests). Number of mice in (C-G): vehicle-treated wt (von Frey/Hargreaves test) n=7/11, RHHH n=15/12, RHRR n=18/19, RRHR n=13/9, RRRH n=11/12, RHHR n=19/10, RHRH n=9/7, RRRR n=9/6, RRHH n=8/19. (**H**) Formalin test. Reduction by DZP (10 mg/kg) in the number of flinches and licking bouts compared to vehicle-treated mice. ***, P<0.001; **, P<0.01; *, P<0.05 (ANOVA followed by Dunnett's *post hoc* test. Flinches, F(3,24)=6.71; licking bouts, F(4,25)=10.7. n=9, 6, 7, and 6 mice, for vehicle-treated wt, and DZP-treated RHRR, RRHR, and RRRH mice, respectively. All data points are mean±SEM.

Table 1: Baseline nociceptive sensitivities of all $GABA_AR$ genotypes under study

genotype	BDZ-sensitive α subunit(-s)	pre-CCI von Frey (g) (number of mice)	pre-CCI Hargreaves (s) (number of mice)	post-CCI von Frey (g) (number of mice)	post-CCI Hargreaves (s) (number of mice)
HHHH (wt)	α1,α2,α3,α5	4.04 ± 0.07 (20)	22.9 ± 0.72 (17)	1.48 ± 0.23 (8)	11.7 ± 1.03 (16)
RHHH	α2,α3,α5	4.41 ± 0.11 (23)	22.6 ± 0.63 (15)	1.52 ± 0.11 (15)	9.68 ± 0.81 (12)
RHHR	α2,α3	4.21 ± 0.12 (26)	23.4 ± 0.57 (15)	1.46 ± 0.09 (20)	10.4 ± 1.15 (10)
RHRH	α2,α5	4.05 ± 0.09 (23)	22.9 ± 0.60 (20)	1.26 ± 0.10 (9)	12.8 ± 1.70 (7)
RRHH	α3,α5	4.23 ± 0.04 (29)	21.0 ± 0.43 (29)	1.52 ± 0.06 (8)	8.54 ± 0.62 (19)
RHRR	α2	4.12 ± 0.06 (32)	22.2 ± 0.53 (32)	1.42 ± 0.11 (18)	10.7 ± 0.72 (19)
RRHR	α3	4.12 ± 0.12 (29)	22.2 ± 0.50 (29)	1.27 ± 0.08 (13)	10.3 ± 1.00 (9)
RRRH	α5	4.32 ± 0.07 (23)	21.5 ± 0.42 (23)	1.67 ± 0.08 (11)	10.2 ± 0.89 (12)
RRRR	none	3.99 ± 0.10 (9)	20.8 ± 1.48 (6)	1.18 ± 0.16 (9)	10.3 ± 1.51 (6)
statistics		$F(8,205) = 1.57$; $P \geq 0.06$	$F(8,177) = 1.93$; $P \geq 0.06$	$F(8,102) = 1.78$; $P \geq 0.16$	$F(8,101) = 1.80$; $P \geq 0.09$

values are given as mean±SEM

ANOVA followed by Dunnett's *post hoc* test with wt (HHHH) as control.

Importantly, mice in which all four BDZ receptors had been point-mutated (RRRR mice) did not show any antihyperalgesic response. We next asked whether targeting of a second or third $GABA_AR$ subtype in addition to α2 would increase antihyperalgesic efficacy and compared antihyperalgesic responses in triple point-mutated mice with those in mice carrying only one or two point-mutated receptors. We found only small and statistically insignificant differences indicating that adding activity at a subtype different from α2 did not significantly increase antihyperalgesia (Fig. 2 F).

The autoradiography data shown in Fig. 1 D suggest a high prevalence of $GABA_ARs$ with two different α subunits ("mixed $GABA_ARs$") in the spinal cord. Because the sum of the four receptor subtypes detected in the triple point-mutated mice greatly exceeded the total number of $[^3H]$flumazenil binding sites in the wt mice, the pharmacological effect of a given $GABA_AR$ subtype may be overestimated when tested in triple point-mutated mice. To address this issue, we compared the level of antihyperalgesia achieved in the triple point-

mutated mice with the loss in antihyperalgesia that occurs after mutation of the same subunit (Fig. 2 G). For both types of sensory tests (Hargreaves and von Frey filament) and for all three α subunits under study, we found that the level of antihyperalgesia achieved in the triple point-mutated mice always exceeded the loss in antihyperalgesia by mutation of the same subunit consistent with the high prevalence of mixed $GABA_ARs$ in the spinal cord. It is important to note that the rank order of antihyperalgesic efficacies was the same in experiments with triple point-mutated mice and in loss-of-function experiments in single and double point-mutated mice, and also identical to those found in a pain model employing chemical nociceptor activation instead of neuropathy (Fig. 2 H).

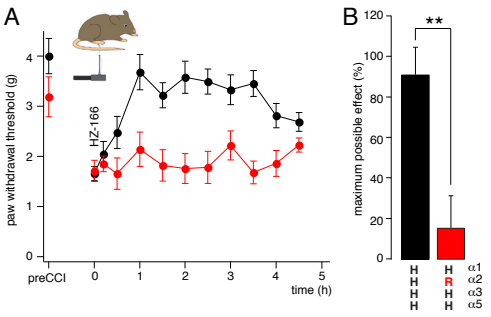

Figure 3. Antihyperalgesia by the non-sedative BDZ HZ-166 occurs through α2GABA$_A$Rs. HZ-166 (16 mg/kg, i.p,) almost completely reversed CCI-induced mechanical sensitization in wt mice (HHHH, black), but not in α2(H101R) mice (HRHH mice, red). (B) Statistical analysis. **, $P<0.01$ (unpaired t-test), n=7 and 8 mice, for wt and α2(H101R) mice. All data points are mean±SEM.

Taken together, these results support a major contribution of α2GABA$_A$Rs to antihyperalgesia in different pain models. However, because all experiments had to be done on an α1 point mutated background to avoid confounding sedation, the relevance of α2GABA$_A$Rs might be overestimated if "pain-relevant" α2GABA$_A$Rs contained also α1 subunits. To address this potential caveat we used HZ-166 (Rivas et al., 2009), a novel benzodiazepine site ligand with an improved α2/α1 selectivity ratio (Fischer et al., 2010) and significant analgesic activity already at non-sedative doses (Di Lio et al., 2011) (Fig. 3). Antihyperalgesia by HZ-166 was almost completely blocked in single α2(H101R) point mutated (HRHH) mice further confirming that the "pain-relevant" $GABA_ARs$ exhibit an α2 pharmacology.

Potential undesired BDZ effects in triple point-mutated mice

We next used the same triple point-mutated mouse approach to investigate non-pain-related effects. In these experiments, we focused on diminished locomotor activity (as a surrogate parameter of sedation), muscle strength, and motor coordination. No significant differences

were observed in the behavior of drug naïve wt and point-mutated mice (Tab. 2). DZP treatment strongly reduced locomotor activity in wt mice and in mice with BDZ-sensitive $GABA_ARs$ of only the α1 subtype (HRRR mice) (Fig. 4 A). No sedative effects were observed in any of the other triple point-mutated mice. Mice with only α2 BDZ-sensitive $GABA_ARs$ (RHRR mice) even showed a strong increase in locomotor activity, which may originate from the anxiolytic effect of DZP occurring through α2$GABA_ARs$ (Löw et al., 2000).

Table 2: Baseline locomotor activity, and performance in the horizontal wire and rotarod tests

genotype	BDZ-sensitive α subunit(-s)	activity counts (number of mice)	horizontal wire performance[1] (number of mice)	rotarod performance[2] (number of mice)
HHHH (wt)	α1,α2,α3,α5	962 ± 114 (14)	92 ± 2.5 (17)	92 ± 5.6 (5)
HRRR	α1	1102 ± 104 (9)	98 ± 1.2 (9)	98 ± 8.2 (6)
RHRR	α2	1132 ± 106 (8)	96 ± 1.2 (8)	81 ± 5.3 (5)
RRHR	α3	1279 ± 114 (10)	99 ± 1.1 (10)	85 ± 7.7 (5)
RRRH	α5	987 ± 90.4 (8)	100 ± 0.0 (8)	84 ± 11 (5)
RRRR	none	1037 ± 115 (9)	99 ± 1.2 (9)	110 ± 13 (5)
statistics[3]		$F(5,52) = 1.16$; $P \geq 0.13$	$F(5,55) = 2.10$; $P \geq 0.70$	$F(5,25) = 1.53$; $P \geq 0.47$

values are given as mean±SEM

[1]horizontal wire performance, expressed as success rate (%)
[2]rotarod performance, expressed as time before fall-off (s)
[3]ANOVA followed by Dunnett's *post hoc* test with wt (HHHH) as control.

Impairment of muscle strength was assessed in the horizontal wire test. Significant muscle relaxation was detected in wt mice and in mice with BDZ-sensitive $GABA_ARs$ of either only the α2 (RHRR mice) or α3 subtype (RRHR mice) (Fig. 4 B). Motor coordination, tested in the rotarod test, was significantly impaired by DZP in mice with only α1 (HRRR mice) and with only α3 BDZ-sensitive $GABA_ARs$ (RRHR mice) (Fig. 4 C). Quadruple point-mutated (RRRR) mice were completely protected from DZP-induced muscle relaxation and motor impairment. They did however show a trend towards reduced locomotor activity (compare Fig. 4 A). We therefore assessed changes in locomotion also after higher DZP doses in the quadruple point-mutated mice and found significant and dose-dependent impairment starting at 30 mg/kg (Fig. 4 D). Unlike α1$GABA_AR$-mediated sedation, impairment of muscle

strength was absent in the quadruple point-mutated mice even at doses ≥ 30 mg/kg. The sedative action of DZP remaining in the quadruple point-mutated mice may be attributed to a low affinity BDZ binding site at α1GABA$_A$Rs described earlier (Drexler et al., 2010, Walters et al., 2000).

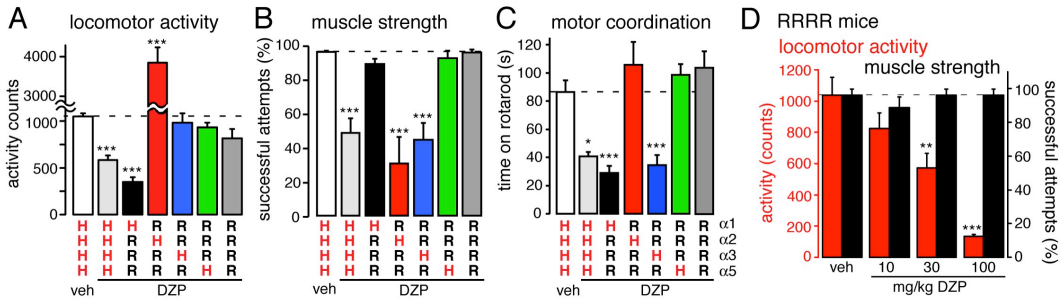

Figure 4. Sedation, muscle relaxation and motor coordination in GABA$_A$R mutated mice.
Effects of DZP (10 mg/kg, p.o.) on locomotor activity in the open field test (**A**), on muscle relaxation in the horizontal wire test (**B**), and on motor coordination in the rotarod test (**C**). ***, P<0.001; *, P<0.05 significant versus vehicle-treated wt (HHHH) mice (ANOVA followed by Dunnett's *post hoc* test). Statistics: locomotor activity F(6,177)=79.5 (n=106, 26, 8, 8, 9, 13, and 13 mice, for vehicle and DZP-treated wt mice, and DZP-treated HRRR, RHRR, RRHR, RRRH, and RRRR mice, respectively). Horizontal wire F(6,165)=44.0 (n=109, 14, 8, 8, 9, 13, and 10 mice). Rotarod F(6,41)=11.5 (n=8, 5, 6, 7, 6, 8, and 8 mice). (**D**) Effects of DZP on locomotor activity and horizontal wire performance in quadruple GABA$_A$R point-mutated (RRRR) mice. ***, P<0.001; **, P<0.05 significant versus vehicle (ANOVA followed by Dunnett's *post hoc* test) F(3,33)=13.4 (locomotor activity), n=9, 13, 8, and 7 mice, for vehicle, and 10, 30, and 100 mg/kg DZP; F(3,30)=0.44; P>0.60 (horizontal wire test), n=9, 10, 8, and 7 mice, for vehicle, and 10, 30, and 100 mg/kg DZP. All data points are mean±SEM.

Our findings on the contribution of α1, α2, and α3GABA$_A$Rs to sedation, anxiolysis, and muscle relaxation confirm previous studies using single GABA$_A$R point mutated mice (Rudolph et al., 1999; Löw et al., 2000; Crestani et al., 2001). The results from our experiments with triple point mutated mice on motor coordination however differ from those obtained with single point mutated mice (Rudolph et al., 1999; Löw et al., 2000). This discrepancy may arise from the involvement of mixed GABA$_A$Rs containing α1 and α3 subunits. We therefore tested whether motor coordination would also be impaired by TP003, an α3GABA$_A$R selective BDZ site agonist (Dias et al., 2005). Two hours after treatment with TP003 (10 mg/kg, p.o.), the time to fall off the rod was similarly decreased in wt and RRHR mice (by 43.9 ± 10.2%, paired t-test P < 0.05, n = 5, in wt mice, and by 38.5 ± 6.4, n = 5, P < 0.05) while HHRH and vehicle treated wt mice showed no impairment (0.7 ± 12.0%, n = 5, P = 0.91, and -4.7±9.2%, n = 5, P = 0.59).

Liability to tolerance development

Another major limitation of classical BDZs is their liability to tolerance development (i.e. the loss activity during prolonged use). We tested whether this tolerance would also occur for the antihyperalgesic actions of BDZs and whether it could be prevented by selectively targeting α2GABA$_A$Rs. To this end, we applied again CCI surgery and treated mice of the different strains for 9 consecutive days with 10 mg/kg DZP or vehicle p.o. once daily. On the 10th day, both groups of mice were split into two subgroups, treated with either 10 mg/kg DZP or vehicle, and mechanical hypersensitivity was monitored for 2 hours after drug application. We first did this experiment in single α1 point-mutated (RHHH) mice to avoid confounding sedation (Fig. 5 A,D). While DZP-naïve mice responded with an almost complete reversal of hyperalgesia, mice chronically pre-treated with DZP showed no antihyperalgesic response. We then performed this experiment in triple point-mutated RHRR mice with only α2 BDZ-sensitive GABA$_A$Rs and found that these mice were completely protected from tolerance development (Fig. 5 B,E). In the latter experiment, the antihyperalgesic response was generally lower than in α1 point-mutated (RHHH) mice. We therefore repeated the first experiment with a lower dose of DZP (3 mg/kg) to exclude the possibility that the absence of tolerance in the RHRR mice was due to a smaller DZP effect. With this lower dose, complete tolerance still developed in RHHH mice (Fig. 5 C,D). These experiments indicate that activity at α3 or α5GABA$_A$Rs was necessary to induce antihyperalgesic tolerance. We therefore tested double point-mutated mice in which α3GABA$_A$Rs or α5GABA$_A$Rs were left BDZ-sensitive in addition to α2GABA$_A$Rs (RHHR and RHRH mice). Complete tolerance developed in RHHR mice, but not in RHRH mice, indicating that activity at α3GABA$_A$Rs was necessary for induction of antihyperalgesic tolerance in mutated mice (Fig. 5E). In this set of experiments, we finally tested whether the same subtype-dependence would also be found for tolerance against α2GABA$_A$R-mediated anxiolysis (Fig. 5F). Unlike antihyperalgesic tolerance, anxiolytic tolerance, measured as increased locomotor activity in the open field, still developed in RHRR mice. This dissociation suggests that tolerance development against antihyperalgesia is not a receptor or cell autonomous process (Jacob et al., 2012) but rather occurs at the circuit level.

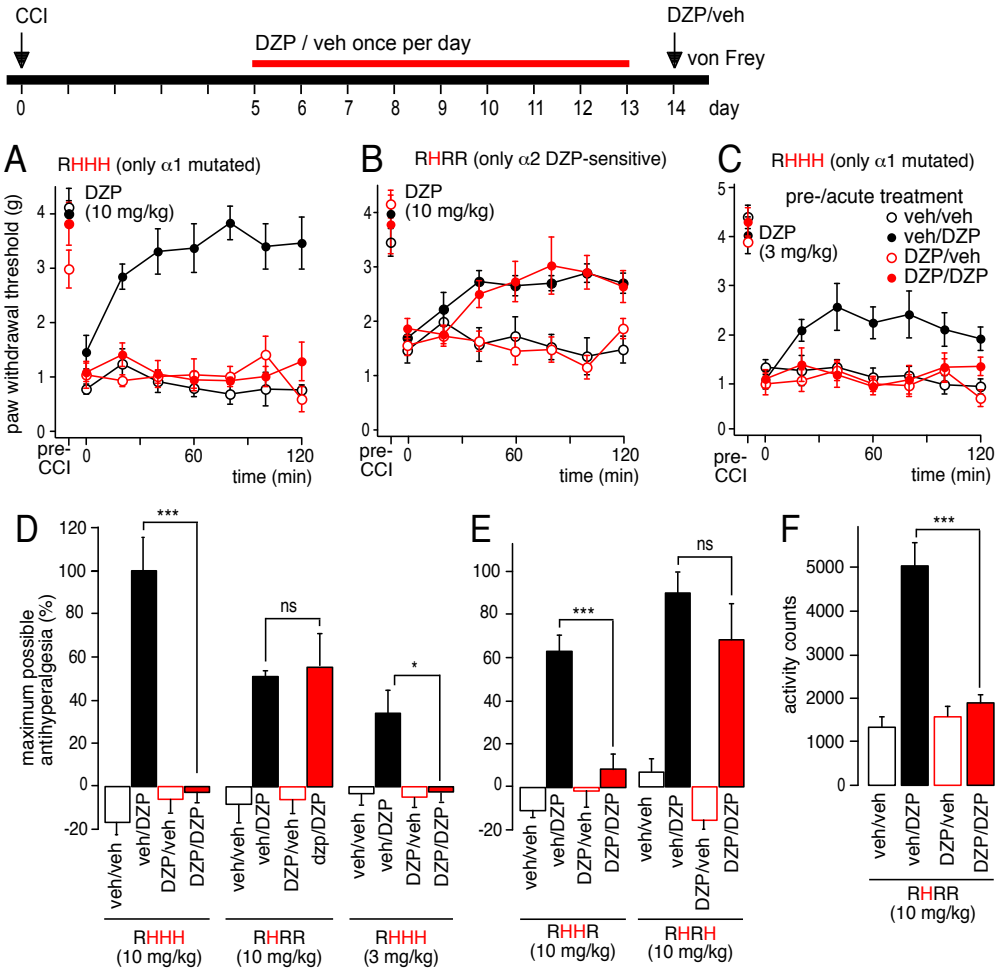

Figure 5. Tolerance liability against the antihyperalgesic effects of DZP.
Starting on day 5 after CCI surgery, mice were treated with DZP or vehicle once daily for 9 consecutive days. On day 10, mice were given either DZP or vehicle, and antihyperalgesic effects were measured for 2 hours. (**A**) Complete loss of antihyperalgesic activity was observed after 9-day DZP treatment (10 mg/kg, p.o.) in RHHH mice. (**B**) Same as (A) but RHRR triple point-mutated mice. These mice were completely protected from tolerance development against DZP-induced antihyperalgesia. (**C**) Same as (A) but lower dose of DZP (3 mg/kg, p.o.). Tolerance in RHHH mice still developed at a lower dose of DZP, which induced an antihyperalgesic effect similar to that of 10 mg/kg in RHRR mice. (**D**) Statistical comparison. Two-way ANOVA for the interaction pretreatment x acute treatment ($F(3,25)=32.5$ (n=5, 8, 7, and 8 mice for veh/veh, veh/DZP, DZP/veh, and DZP/DZP groups, respectively); $F(3,25)=0.014$ (n=7, 8, 7, and 6); $F(3,24)=5.97$ (n=8, 7, 7, and 6), for data shown in A, B, and C). Maximum antihyperalgesic activity was calculated for the interval between 80 and 120 min after DZP administration. (**E**) Double $GABA_AR$ point-mutated mice (RHHR and RHRH), in which $\alpha3GABA_AR$ or $\alpha5GABA_AR$ were left BDZ-sensitive in addition to $\alpha2GABA_AR$. Tolerance development required the additional presence of DZP-sensitive $\alpha3GABA_AR$. Two-way ANOVA for the interaction pretreatment x acute treatment. RHHR mice: $F(3,22)=22.1$ (n=6, 6, 7, and 6 mice for for veh/veh, veh/DZP, DZP/veh, and DZP/DZP groups, respectively); RHRH mice: $F(3,21)=0$ (n=6 for all groups). (**F**) Unlike antihyperalgesic effects, anxiolytic effects of DZP were still susceptible to tolerance development in RHRR mice after 9-day treatment with DZP (10 mg/kg, p.o.). Two-way ANOVA $F(3,26)=28.3$ (n=8, 7, 7, and 7) for the interaction pretreatment x acute treatment. ***, $P=0.001$; *, $P=0.05$. All data points are mean±SEM.

Why classical BDZs lack clinically relevant analgesic properties

Our present results and previous pain studies employing subtype-selective BDZ site agonists in rodents (Knabl et al., 2008; Di Lio et al., 2011; Knabl et al., 2009; Hofmann et al., 2012; Nickolls et al., 2011; Munro et al., 2008) contrast with the lack of a clear analgesic or antihyperalgesic action of classical BDZs in human patients (Richards et al., 2012; Corrigan et al., 2012). Apart from species differences and differences between disease models and actual disease in human patients, we found one possible explanation particularly worth studying. The doses and the degrees of receptor activation required for a relevant effect might be significantly higher in case of antihyperalgesia than of sedation. As a consequence, antihyperalgesia would occur in patients only at doses already inducing strong sedation.

The availability of the triple point-mutated mice allowed us to directly compare the doses and levels of receptor occupancy at the relevant $GABA_AR$ subtypes and sites. For these experiments, we chose midazolam (MDZ) as a second classical BDZ in addition to DZP. First, we determined for both drugs their $\alpha2/\alpha1$ selectivity profiles in electrophysiological experiments on recombinant $GABA_ARs$. As expected we found that DZP potentiated $\alpha1/\beta2/\gamma2$ and $\alpha2/\beta3/\gamma2$ $GABA_ARs$ with similar efficacy and potency (Fig. 6A and Tab. 3).

By contrast, MDZ potentiated $\alpha1/\beta2/\gamma2$ $GABA_ARs$ more than twice as much as $\alpha2/\beta3/\gamma2$ $GABA_ARs$ (Fig. 6B and Tab. 3). We then verified that the H → R point mutation blocked not only DZP effects but also MDZ binding and $GABA_AR$ potentiation (Fig. 6C,D; see also Tan et al., 2010). Next, we compared the dose-dependency of DZP- and MDZ-induced antihyperalgesia in mice with only $\alpha2$ BDZ-sensitive $GABA_ARs$ (RHRR mice) with that of DZP- and MDZ-induced sedation in mice with only $\alpha1$ BDZ-sensitive $GABA_ARs$ (HRRR mice). We found that half maximal sedation occurred already at a doses of 0.33 ± 0.05 mg/kg and of 0.52 ± 0.11 mg/kg (mean ± SD) for DZP and MDZ, respectively, while half maximal antihyperalgesia required 8 ± 6 mg/kg (DZP) and 10.4 ± 2.0 mg/kg (MDZ). A rightward shift of the response curve was also observed when the degrees of receptor occupancy required for antihyperalgesia and for sedation were compared. Half maximal sedation was reached when DZP had bound 47 ± 6% brain $\alpha1GABA_ARs$, while half maximal antihyperalgesia required 71 ± 2% occupancy at spinal $\alpha2GABA_ARs$. In case of MDZ, the required receptor occupancies were even further apart (24 ± 2% of brain $\alpha1GABA_ARs$ and 71 ± 2% of spinal $\alpha2GABA_ARs$) consistent with the even less favorable $\alpha2/\alpha1$ selectivity ratio of MDZ.

These data indicate that, when applied to wt mice, the DZP and MDZ doses needed for half maximal antihyperalgesia induce an almost complete (about 95%) reduction in locomotor activity, while at non-sedative doses both BDZs would not induce significant antihyperalgesia. Dose-limiting sedation is therefore the most likely reason for the absence of a relevant antihyperalgesic activity of classical, non-selective BDZs in human patients.

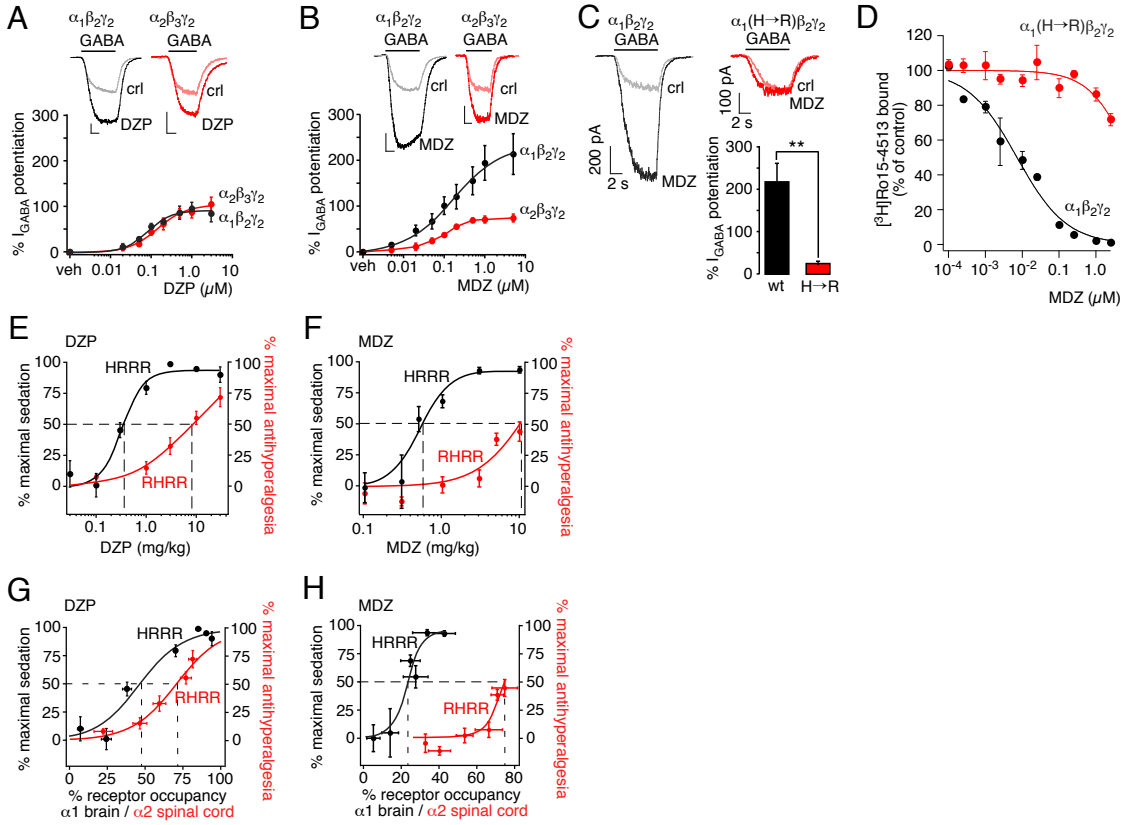

Figure 6. Activity of DZP and MDZ at α1 and α2GABA$_A$Rs, and PK/PD modelling.
(**A,B**) Potentiation of recombinant α1β2γ2 and α2β3γ2 GABA$_A$R currents by 1 μM DZP (A) and 1 μM MDZ (B). GABA concentration was EC$_5$ (5 μM for α1β2γ2 and 2 μM for α2β3γ2). n=6 for both drugs and all concentrations. Scales bars, 200 pA, 2 s. (**C,D**) The H101R mutation prevented potentiation of α1β2γ2 GABA$_A$R currents by MDZ (**, P<0.01, n=5, unpaired t-test) (C) and binding of MDZ to α1β2γ2 GABA$_A$Rs (n=6 for all concentrations in wt and mutated receptors) (D). (**E-H**) α1GABA$_A$R-mediated sedation and α2GABA$_A$R-mediated antihyperalgesia by DZP and MDZ. Sedation (black symbols) in HRRR mice was assessed in the open field test and expressed as percent maximum possible effect determined from the reduction in activity compared to vehicle-treated mice of the same genotype. Antihyperalgesia (red symbols) in RHRR mice was determined as the increase in mechanical withdrawal thresholds compared to pre-drug values one week after CCI surgery. (**E,F**) Dose-response relationships. Data were fitted to Hill's equation with baseline fixed at 0. Sedation and antihyperalgesia were determined between 15 and 90 min after oral DZP (E) or between 15 and 60 min after oral MDZ (F). Mice were killed immediately afterwards (i.e. at the time point of maximal effects), and brains and spinal cords were removed for further analyses. (**G,H**) Dependence on receptor occupancy (RO). (**G**) Sedation versus RO in brains of HRRR mice treated with DZP (0.03, 0.1, 0.3, 1, 3, 10, 30 mg/kg, n=6 mice for all doses). Antihyperalgesia versus RO in lumbar spinal cords of RHRR mice treated with 0.1, 1, 3, 10, or 30 mg/kg DZP, n=6 mice for all doses. (**H**) Same as G but MDZ. Sedation 0.1 (n=6 mice), 0.3 (7), 0.5 (7), 1.0 (7), 3 (6), 10 (4) mg/kg. Antihyperalgesia 0.1 (n=3), 0.3 (5), 1.0 (9), 3 (8), 5 (7), and 10 (7) mg/kg MDZ. Data were fitted to sigmoidal functions. Data shown in E and G (on DZP) and in F and H (on MDZ) are from the same two groups of mice. All data points are mean±SEM.

Table 3: PK/PD parameters[1] of DZP and MDZ

	DZP		MDZ	
	a1/b2/g2	a2/b3/g2	a1/b2/g2	a2/b3/g2
EC_{50} (μM)	0.081 ± 0.020	0.15 ± 0.04	0.17 ± 0.18	0.092 ± 0.019
E_{max} (% pot I_{GABA})	91.6 ± 7.7	103 ± 11	235 ± 43	75.8 ± 4.7
n_H	1.50 ± 0.60	1.20 ± 0.35	0.73 ± 0.26	1.23 ± 0.29
a2/a1 selectivity[2]	1.25		0.32	
	sedation	analgesia	sedation	analgesia
ED_{50} (mg/kg)	0.33 ± 0.05	7.6 ± 0.6	0.52 ± 0.11	10.4 ± 2.0
E_{max} (% MPE)	93.6 ± 4.5	100 (fixed)	92.2 ± 10.4	100 (fixed)
n_H	2.0 ± 0.64	0.75 ± 0.19	2.28 ± 0.98	1.42 ± 0.40
ED_{50} (sedation/analgesia)	0.043		0.050	
	sedation	analgesia	sedation	analgesia
RO_{50} (%)	46.9 ± 5.5	70.9 ± 1.7	23.7 ± 2.2	76.5 ± 1.8
E_{max}	97.9 ± 7.1	100 (fixed)	94.7 ± 11.8	100 (fixed)
rate	14.1 ± 4.1	14.9 ± 2.0	4.14 ± 2.10	5.01 ± 2.11
RO_{50} (sedation/analgesia)	0.66		0.31	

[1] values are mean±SD
[2] E_{max} (a2/b3/g2) / E_{max} (a1/b2/g2)
For number of mice per group see figure 6.

Discussion

In the present study we have employed triple $GABA_AR$ point-mutated mice to characterize the pharmacological actions expected from yet-to-be-developed subtype-selective BDZ binding site agonists. The present study can be viewed as a "restriction-of-function" approach ("what effect of DZP remains when only a single $GABA_AR$ subtype is targeted?") as opposed to previous loss-of-function studies in single $GABA_AR$ point-mutated mice ("which effects of DZP are lost or reduced when activity at one $GABA_AR$ subtype is abolished compared to DZP-treated wt mice?"). With respect to sedation, anxiolysis, and the muscle relaxant action, the present study confirms previous results obtained with single point-mutated mice: sedative actions of BDZs occur through $\alpha1GABA_AR$ (McKernan et al., 2000; Rudolph et al., 1999), anxiolytic effects through $\alpha2GABA_ARs$ (Löw et al., 2000), and muscle relaxation through $\alpha2$ and $\alpha3GABA_AR$ (Crestani et al., 2001).

Discrepancies between results from restriction-of-function and loss-of-function approaches were observed for impairment of motor coordination. Our study shows that undesired impairment of motor coordination is caused by DZP in HRRR and RRHR mice (i.e. it is evoked when either $\alpha1$ or $\alpha3GABA_ARs$ are specifically targeted), while previous reports in the respective single point-mutated mice failed to provide evidence for an involvement of these $GABA_AR$ subtypes (Rudolph et al., 1999; Löw et al., 2000). This discrepancy is

consistent with the idea that DZP impairs motor coordination through mixed $GABA_ARs$ containing an α1 and an α3 subunit (α1/α3$GABA_ARs$). Immunohistochemical analyses have shown that co-expression of α1 and α3 $GABA_AR$ subunits occurs in subsets of central neurons (Fritschy & Möhler, 1995; Bohlhalter et al., 1996), and biochemical data have provided direct evidence for the presence of different α subunits within the same $GABA_AR$ complex (Benke et al., 2004). In particular α3 containing $GABA_ARs$ occur mainly as mixed α1/α3$GABA_ARs$ (Araujo et al., 1996; Benke et al., 2004). Furthermore, biochemical data also suggest that in mixed $GABA_ARs$ with one point-mutated α subunit the non-point-mutated (wt) subunit has a higher probability for interaction with the γ2 subunit than the point-mutated α subunit (Benke et al., 2004; Balic et al., 2009). Results from loss-of-function analysis in single point-mutated mice and from restriction-of-function analyses in triple point-mutated mice should therefore be seen as lower and upper limits for the contribution of a given $GABA_AR$ subtype to an *in vivo* drug action. Conversely, the lack of sedation in α1 point-mutated mice (McKernan et al., 2000; Rudolph et al., 1999) and of anxiolysis in α2 point-mutated mice (Löw et al., 2000) suggests that these actions occur through $GABA_ARs$ with only a single type of α subunit. In line with this conclusion is that the majority of $GABA_ARs$ in the brain are pure α1$GABA_ARs$ (α1/α1$GABA_ARs$) (Benke et al., 2004). Similarly, BDZ-mediated anxiolysis occurs through pure α2$GABA_ARs$ with α2/α2$GABA_ARs$ being the single most prevalent isoform of α2 containing $GABA_ARs$ at supraspinal sites (Benke et al., 2004).

As demonstrated by our autoradiography experiments (compare Fig. 1 D,E), mixed $GABA_ARs$ make up the vast majority of $GABA_ARs$ in the spinal cord. Following the model described above, one would expect that in case of mixed $GABA_ARs$ the single point mutation approach would yield smaller effects than the triple point mutation strategy. For instance, in case of an αx/αy$GABA_AR$ neither the αx single point mutation nor the αy single point mutation would abolish BDZ sensitivity, but both triple point-mutated mice with only αx or only αy remaining BDZ sensitive would yield full behavioral effects. Results for antihyperalgesia obtained in the present study were consistent with a contribution of mixed $GABA_ARs$ to antihyperalgesia. For all three contributing α subunits, the loss-of-function obtained through point mutation of one α subunit was smaller than the restriction-of-function in the respective triple point-mutated mouse. The discrepancies were particularly large for α3 in mechanical sensitization and for α5 in heat hyperalgesia suggesting that these effects occur mainly through mixed $GABA_ARs$. Importantly, the rank orders of antihyperalgesic efficacy

were the same for the restriction-of-function and loss-of-function approaches with α2>α5>α3 for mechanical sensitization and α2>α3>α5 for heat hyperalgesia and chemical nociception. The same rank order of efficacies has also been reported previously for antihyperalgesia using local spinal injections in single $GABA_AR$ point mutated mice (Knabl et al., 2008). Our present results therefore corroborate the critical importance of α2$GABA_ARs$ as targets for antihyperalgesia.

Our experiments on the quadruple point-mutated mice indicate that antihyperalgesia, anxiolysis, muscle relaxation and impairment of motor coordination occur through the high affinity BDZ binding site formed by the α/γ interface. Among the DZP actions assessed here, only sedation by high doses of DZP occurred in quadruple point-mutated mice. This result is consistent with previous electrophysiological data, which suggested the presence of a low-affinity BDZ binding site in α1$GABA_ARs$ contributing to the anesthetic actions of BDZs (Walters et al., 2000).

Most BDZ effects quickly diminish during prolonged treatment (i.e. they undergo fast tolerance development). In the present study, we show that this is also the case for antihyperalgesia. Previous studies with the non-sedative BDZ site ligands L-838,417 and HZ-166 showed that these compounds have strongly reduced liabilities to tolerance development (Knabl et al., 2008; Di Lio et al., 2011; Hofmann et al., 2012). It has however not been possible to attribute this reduced tolerance to generally reduced agonistic activity or to improved subtype specificity. Our present study shows that tolerance can be avoided when only α2$GABA_ARs$ are targeted, even with compounds that exert full agonistic activity. Additional experiments in RHRH and RHHR mice exclude α5$GABA_ARs$ and suggest that activity at α3$GABA_ARs$ is required for tolerance induction. However, because all these experiments were done in mice carrying the H → R point mutation in the α1 subunit, we cannot exclude that tolerance occurs through mixed α1/α3$GABA_ARs$, which in the wt situation may exhibit an α1 pharmacology. Such a scenario would explain why compounds with activity at α3$GABA_ARs$ but absent or reduced activity at α1$GABA_ARs$ lack liability to tolerance (Knabl et al., 2008; Di Lio et al., 2011; Hofmann et al., 2012). Interestingly, tolerance against the anxiolytic action of DZP was retained in triple point-mutated RHRR mice with only α2$GABA_ARs$ remaining BDZ-sensitive. This difference suggests that tolerance development against the different BDZ effects involves activity at distinct subunits and possibly different mechanisms. Cell or receptor autonomous processes, which have been

proposed for the desensitization of hippocampal α2GABA$_A$Rs (Jacob et al., 2012), are unlikely to be responsible for the tolerance against antihyperalgesia.

Addictive (reinforcing) properties of classical BDZs are another area of concern that has not been addressed in the present study. Reduced BDZ-induced reward facilitation has been reported in mice carrying the H → R point mutation in either the α1, α2, or α3GABA$_A$R subunits (Engin et al., 2014; Reynolds et al., 2012). Preference for MDZ in a two-bottle choice paradigm was absent in α1 (Corrigan et al., 2012; Engin et al., 2014) and α2 point-mutated mice (Engin et al., 2014), suggesting that the simultaneous positive modulation of both the α1 and α2GABA$_A$R are required for reinforcement. Tan et al. (ref. 32) reported that α1GABA$_A$Rs on GABAergic neurons in the ventral tegmental area (VTA) are required for MDZ-induced neuronal plasticity strengthening glutamatergic excitation in the VTA. These findings suggest that activity at more than one GABA$_A$R subtype is necessary for BDZ reinforcement. α2-selective BDZ site agonists might therefore be largely devoid of addictive properties.

Previous studies have provided evidence that activation of supraspinal GABA$_A$Rs might enhance pain and counteract spinal antihyperalgesia, e.g. through the inhibition of descending antinociceptive fiber tracts (Tatsuo et al., 1999; Luger et al., 1995; Harris & Westbrook, 1995). In our study, we did not observe any pronociceptive actions of systemically administered DZP. If such pronociceptive actions were relevant in the pain models used here, they would occur through α1GABA$_A$Rs whose functions in nociception were not addressed in the present study. Any such effects would be avoided by α1-sparing BDZ site agonists.

The availability of the triple GABA$_A$R point-mutated mice permitted a direct pharmacokinetic/pharmacodynamic comparison of α1-mediated sedation and α2-mediated antihyperalgesia in the absence of confounding behavioral effects from other GABA$_A$R subtypes. In case of DZP, ED_{50} values for sedation and antihyperalgesia differed by a factor of more than 20, and a 50% higher degree of receptor occupancy was needed for α2-mediated antihyperalgesia compared to α1-mediated sedation. New subtype-selective agents will therefore have to have a high degree of α2 over α1 selectivity in order to achieve clinically relevant antihyperalgesia in the absence of sedation. These data also indicate that dose-limiting sedation most likely underlies the lack of clinically relevant antihyperalgesic effects of presently used (non-selective) BDZs. This is consistent with a recent clinical study in human volunteers showing weak antihyperalgesic effects at doses that caused only mild sedation (Vuilleumier et al., 2013).

Which $GABA_AR$ subtype should be targeted for an optimal benefit-risk ratio in pain treatment? Both the present restriction-of-function and loss-of-function experiments on systemic BDZ administration and previous experiments with local spinal injections and single $GABA_AR$ point-mutated mice (Knabl et al., 2008) attribute the highest antihyperalgesic efficacy to $\alpha2GABA_ARs$. The present study has shown that adding activity at $\alpha3GABA_ARs$ or $\alpha5GABA_ARs$ increases antihyperalgesic efficacy only moderately or not at all. Because it is at present not known how the different mixed $GABA_ARs$ respond to subtype-selective agents, a conservative prediction should be made on the basis of the results obtained from triple point-mutated mice. These experiments indicate that sedation is exclusively due to activation $\alpha1GABA_ARs$, which is also consistent with studies using the subtype selective agonist TPA023B that completely lacks agonistic activity at $\alpha1GABA_ARs$ as well as sedation in humans (Atack et al., 2011). The present experiments also showed that impaired motor coordination involves $\alpha1GAB_ARs$ and/or $\alpha3GABA_ARs$. None of the undesired effects investigated in the present study could be attributed to $\alpha5GABA_ARs$. However, cognitive impairment by non-selective BDZ likely originates from activity at $\alpha5GABA_ARs$, as $\alpha5GABA_AR$-selective inverse agonists enhance cognitive capabilities (Dawson et al., 2006). Subtype-selective BDZ agonists targeting only $\alpha2GABA_ARs$ should therefore have the best benefit-risk ratio. They will produce pronounced antihyperalgesia in the absence of sedation and will not interfere with motor coordination and should not lose antihyperalgesic activity during prolonged treatment. They will however have anxiolytic properties (at least during the beginning of the treatment) and muscle relaxant effects. Both of these actions may be beneficial in chronic pain patients. Because the side-effects studied here are relevant for other indications, we expect that our present findings will benefit not only the development of innovative subtype-selective BDZs as analgesics but also as drugs for the treatment of several prevalent psychiatric diseases (Rudolph & Möhler, 2014).

Methods

Mice

Experiments were performed in wt mice, and in homozygous single, double, triple and quadruple (H→R) $GABA_AR$ point-mutated mice, i.e. in mice expressing different combinations of BDZ-sensitive and BDZ-insensitive $GABA_AR$ α subunits. All mice were of the same genetic background (129X1/SvJ). Double, triple and quadruple point-mutated mice were generated by cross breeding single point-mutated mice described earlier (Rudolph et al., 1999; Löw et al., 2000; Crestani et al., 2002).

Autoradiography

The distribution of BDZ-sensitive $GABA_AR$ subtypes in the lumbar spinal cord was examined in 16 µm thick horizontal sections, which were cut from fresh-frozen spinal cords. Sections were incubated with 5 nM [^{3}H]flumazenil (50 Ci/mmol) or 9 nM [^{3}H]Ro15-4513 (22.7 Ci/mmol) diluted in 50 mM Tris pH 7.4 for 120 min on ice. After washing three times for 20 s in ice-cold buffer, sections were dried and exposed along with [^{3}H]-standards to a tritium-sensitive phosphoimaging screen (Cyclone Storage Phosphor Screen, Perkin Elmer). Quantification was done using the Optiquant software (Perkin Elmer). Non-specific binding was assessed by co-incubating 10 µM clonazepam ([^{3}H]flumazenil binding) or 10 µM flumazenil ([^{3}H]Ro15-4513 binding).

Immunohistochemistry

The localization of $GABA_AR$ subunits was visualized on 40 µm thick lumbar spinal cord cryosections by DAB immunoperoxidase staining on sections from aCSF perfused mice postfixed for 90 min in 4% PFA (without picric acid) (Paul et al., 2012). Antibodies were home-made subunit-specific antisera (Paul et al., 2012). Final dilutions were 1:20,000 (α1), 1:1,000 (α2), 1:10,000 (α3), 1:3,000 (α5) and 1:10,000 (γ2). Images were taken with a bright field light microscope connected to a digital camera, processed with AxioVision Rel. 4.5 software where an intensity-gradient false-color filter was applied.

Behavioral experiments

All behavioral experiments were performed in 7 – 12 week old female and male mice. Care was taken to ensure equal numbers of female and male mice. All behavioral experiments were made by a male experimenter, blinded either to the genotype of the mice or to their

treatment with drug or vehicle. Permission for animal experiments was obtained from the Veterinäramt des Kantons Zürich (license numbers 135/2009 and 126/2012).
DZP (suspended in 0.9% saline, 1% Tween80) and MDZ (dissolved in 0.9% saline pH 3.0) were applied orally in all experiments. HZ-166 (suspended in 0.5% methyl cellulose) was given intraperitoneally (i.p.).
Neuropathic pain was evoked through CCI (Bennett & Xie, 1988) of the left sciatic nerve proximal to the trifurcation with three loose (5-0 silk) ligatures. Mice, which showed signs of paralysis or which did not develop significant hypersensitivity, were excluded from subsequent experiments. Effects of DZP and MDZ (p.o. in 0.9% saline, 1% Tween80) on thermal and mechanical hyperalgesia were assessed one week after surgery. Heat hyperalgesia was quantified as the change in the latency of paw withdrawal evoked by exposure of the plantar side of one hindpaw to a defined radiant heat stimulus. Mechanical hyperalgesia was assessed with electronic von Frey filament #7 (IITC, Woodland Hills, CA). Three to four measurements were made for each time point and animal for both heat and mechanical hyperalgesia. Percent maximal possible effect (%MPE) was calculated as follows:

$\%MPE(t) = (E(t)-E_{predrug}))/(E_{preCCI}-E_{predrug})$; E(t), paw withdrawal thresholds or latency at time point t. $E_{predrug}$, E after CCI surgery but before DZP application; E_{preCCI}, E baseline before CCI surgery.

Formalin (4 %, 20 µl) was injected subcutaneously into the plantar surface of the left hindpaw. Flinches and licks of the injected paw were counted for one hour in 5 min intervals starting immediately after formalin injection. DZP was administered p.o. 60 min before formalin.

Locomotor activity was measured in an open field arena (10 cm diameter) equipped with four pairs of light beams and photosensors. Mice were placed into the arena 75 min before DZP application. Locomotor activity was analyzed for the time interval between 20 and 165 min after DZP administration.

Motor control was assessed with a rotarod accelerating from 4 rpm to 40 rpm within 5 min. Sixty min after DZP administration mice were placed onto the rotarod. Five measurements were taken per mouse.

To assess muscle relaxation, mice were placed with their forepaws onto a metal horizontal wire placed 20 cm above ground. Successes and failures to grab the wire with at least one hindpaw were recorded between 60 and 180 min after DZP administration.

Electrophysiology

The effects of DZP and MDZ on currents through recombinant $GABA_A$Rs were studied in HEK293 cells (ATCC) transiently expressing $GABA_A$Rs. HEK293 cells were transfected using lipofectamine LTX (Paul et al., 2014). To ensure expression of the γ2 subunit (required for modulation of $GABA_A$Rs by BDZs) in all recorded cells, we transfected cells with a plasmid expressing the γ2 subunit plus eGFP from an IRES, and only selected eGFP-positive cells for recordings. The transfection mixture contained (in μg): 1 α1, 1 β2, 3 γ2/eGFP (used as a marker of successful transfection) or 1 α2, 1 β3, 3 γ2/eGFP. Recordings were made 18 – 36 hrs after transfection. Whole-cell patch-clamp recordings of GABA-evoked currents were made at room temperature (20 – 24°C) and at a holding potential of -60 mV. Recording electrodes were filled with solution containing (in mM): 120 CsCl, 10 EGTA, 10 HEPES (pH 7.40), 4 $MgCl_2$, 0.5 GTP and 2 ATP. The external solution contained (in mM): 150 NaCl, 10 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES (pH 7.4), and 10 glucose. GABA was applied to the recorded cell using a manually controlled pulse (4 - 6 s) of a low sub-saturating GABA concentration (EC_5). EC_5 values of GABA were determined for wt α1β2γ2, wt α2β3γ2 and mutant α1(H101R)β2γ2 receptors. EC_{50} values and Hill coefficients (n_H) were obtained from fits of normalized concentration–response curves to the equation $I_{GABA} = I_{max}\,[GABA]n_H / ([GABA]n_H + [EC_{50}]n_H)$. I_{max} was determined as the average maximal current elicited by a concentration of 1 mM GABA. DZP and MDZ dissolved in DMSO and subsequently diluted with recording solution were co-applied together with GABA without preincubation.

[^{3}H]Ro 15-4513 binding assay to recombinant $GABA_A$Rs

HEK293 cells were maintained in DMEM supplemented with 10% FBS and seeded to a density of 500,000 cells onto poly-lysine-coated 100 mm culture dishes one day before transfection. Cells were transfected with plasmids containing the subunit combination α1β2γ2 or α1(H101R)β2γ2 (7 mg total DNA, ratio 1:1:2) using jetPEI transfection reagent (Polyplus-transfection). Twenty-four hours after transfection, HEK293 cells were harvested in PBS. HEK293 cells were homogenized in 10 mM Tris pH 7.5, protease inhibitor cocktail (complete Mini, Roche Applied Science). Aliquots of the homogenate prepared from

HEK293 cells expressing the α1β2γ2 or α1(H101R)β2γ2 subunit combination were incubated with increasing concentrations of MDZ and 6.3 nM [^{3}H]Ro15-4513 (22.7 Ci/mmol, PerkinElmer) in a total volume of 200 µl for 90 min on ice. Subsequently, the samples were filtered onto glass fiber filters using a 12-channnel semiautomated cell harvester (Scatron) and washed with ice-cold buffer (10 mM Tris-HCl pH 7.4). Non-specific [^{3}H]Ro15-4513 binding was measured using 10 µM flumazenil. The radioactivity of the filters was determined by liquid scintillation counting using a Tricarb 2500 liquid scintillation analyzer.

Receptor occupancy

Mice were killed immediately after completion of the behavioral tests, i.e. 90 min after DZP administration and 60 min after MDZ administration. Brains and spinal cords were removed and kept frozen until further processing. Percentage of receptors occupied by DZP or MDZ was determined *ex vivo* with [^{3}H]flumazenil as radioligand (Li et al., 2006). Frozen brains or lumbar spinal cords were rapidly homogenized in 20 volumes 10 mM Tris pH 7.4, 100 mM KCl, and 100 µl aliquots were immediately added to 400 µl ice-cold buffer containing 4 nM [^{3}H]flumazenil. After 30 s of incubation, samples were filtered onto Whatman GF/C glass-fiber filters and washed 3 times with 4 ml ice-cold buffer. Radioactivity retained on the filters was determined by scintillation counting. To determine non-specific [^{3}H]flumazenil binding, mice were used that had been injected i.p. with 5 mg/kg bretazenil (dissolved in PEG300) to fully occupy all BDZ-binding sites (100% receptor occupancy (Li et al., 2006)).

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Author contributions

WTR performed all experiments, except the electrophysiological measurement, and analyzed the data. DB performed and analyzed the radio ligand binding experiments. MAA performed and analyzed the electrophysiology experiments. UR helped design the experiments. HUZ designed experiments, analyzed data and wrote the manuscript. All authors made comments on the manuscript.

Competing financial interest

The authors declare no competing financial interest.

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N-DESMETHYL CLOBAZAM IS AN α2 PREFERRING BENZODIAZEPINE THAT PRODUCES ANTIHYPERALGESIA IN MICE IN THE ABSENCE OF OBVIOUS SEDATION*

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Abstract

Data obtained in genetically modified mice suggest that benzodiazepine-site ligands with improved selectivity for α2-subtype $GABA_A$ receptors are potentially useful in the treatment of pathological pain syndromes. Compounds available for preclinical tests support that this concept works in rodents but none of these compounds has so far been approved for use in humans precluding proof-of-concept studies in human volunteers or pain patients. We have recently proposed that N-desmethyl clobazam (NDMC), the main metabolite of clobazam (CBZ), contributes to the antihyperalgesia observed in mice after administration of CBZ. In the present study we compared the *in vitro* $GABA_AR$ subtype specificity of NDMC and CBZ and compared their propensity to sedation. We found that NDMC has higher efficacy at α2-$GABA_AR$ and weaker sedative effects than CBZ. NDMC exerted significant antihyperalgesic effects in the chronic constriction model of neuropathic pain at doses between 3 and 30 mg/kg body weight. At 30 mg/kg NDMC completely reversed heat and mechanical hyperalgesia. This antihyperalgesic response was completely lost in quadruple point mutated mice in which all high-affinity benzodiazepine sites had been rendered insensitive. At doses up to 30 mg/kg NDMC did not cause a significant reduction in muscle strength and did not impair motor coordination. Our data suggest that NDMC might be a useful tool compound to assess potential analgesic or antihyperalgesic effects of improved benzodiazepine site ligands in human volunteers or pain patients.

Introduction

Chronic pain is a medical condition that is often resistant to currently available pharmacotherapy. In particular neuropathic pain does not respond well to the majority of conventional analgesics and drugs that are effective often elicit intolerable side-effects prompting for the discontinuation of treatment. The identification of novel drug targets based on disease mechanisms and the developments of new analgesic drugs targeting the mechanisms of pain pathology should offer the opportunity to improve the current situation.

Many chronic pain states, at least in animal models, are accompanied by diminished synaptic inhibition at the spinal cord level (Zeilhofer et al., 2012). Their potential relevance as a disease mechanism is supported by rodent experiments showing that a blockade of spinal GABA and glycine receptors induces pain behaviors (Beyer et al., 1985; Roberts et al., 1986; Sivilotti & Woolf, 1994). Conversely, spinally applied diazepam, which enhances GABAergic inhibition, reverses pathologically increased pain sensitivity in rodent models of chronic inflammatory and neuropathic pain (Knabl et al., 2008a). This action occurs through the α2-subtype of $GABA_A$ receptors (α2-$GABA_A$Rs; (Knabl et al., 2008b)). Genetically mutated (triple $GABA_A$ receptor point mutated mice) in which all $GABA_A$R subtypes had been rendered diazepam (DZP)-insensitive except for the α2-$GABA_A$Rs show pronounced antihyperalgesia in response to systemic DZP administration but do not exhibit signs of sedation or impaired motor coordination (Ralvenius et al., 2015). The observation that the different desired and unwanted effects of classical benzodiazepines are mediated by distinct $GABA_A$R subtypes has stimulated the development of benzodiazepine site agonists with improved subtype selectivity (Rudolph & Knoflach, 2011). Because many of the typical side effects of classical benzodiazepines such as sedation, addiction, and motor impairment depend on activation of α1-$GABA_A$Rs (Rudolph et al., 1999; Tan et al., 2010; Ralvenius et al., 2015) most efforts aimed at the development of benzodiazepine site agonists with reduced activity at α1-$GABA_A$Rs. Such compounds were also evaluated in different rodent pain models (Knabl et al., 2008b; Knabl et al., 2009; Munro et al., 2009; Di Lio et al., 2011; Nickolls et al., 2011; Hofmann et al., 2012; Reichl et al., 2012; Paul et al., 2014), reviewed in (Zeilhofer et al., 2012). These studies have provided proof-of-concept evidence that the results obtained in genetically modified mice translate into antihyperalgesic efficacy of novel benzodiazepine site agonists with improved subtype selectivity.

However, given recent concerns about the predictive value of animal and, in particular, rodent pain models in pain research (Tappe-Theodor & Kuner, 2014), it appears important to obtain proof-of-concept data on the translatability of these findings to human pain patients. Classical benzodiazepine site agonists such as clonazepam and clobazam have been shown to exert weak analgesic effects at mildly sedating doses (Vuilleumier et al., 2013; Besson et al., 2015). Our recent preclinical study comparing antihyperalgesic and sedative effects of DZP and midazolam in genetically modified mice suggests that dose limiting sedation is the major reason for the absence of clinically relevant analgesic effects in humans (Ralvenius et al., 2015). However, based on these data species differences cannot be excluded. None of the recently developed benzodiazepine-site ligands with improved selectivity for α2-$GABA_A$Rs has so far been approved for use in human patients (Rudolph & Knoflach, 2011). Therefore these compounds are currently not readily available for proof-of-concept studies in human patients. We therefore evaluated alternative possibilities. In a previous preclinical pharmacokinetic/ pharmacodynamic (PK/PD) study on possible antihyperalgesic effects of clobazam, a clinically used benzodiazepine with less sedative effects, we found that antihyperalgesic effects correlated better with plasma levels of the main clobazam metabolite N-desmethyl clobazam (NDMC) than with plasma levels of the parent compound (Besson et al., 2013). In the present study we have systematically evaluated the subtype selectivity of NDMC in human recombinant $GABA_A$Rs, and its antihyperalgesic effects in a mouse model of neuropathic pain and for different potential unwanted effects. We found that NDMC has a better α2/α1-$GABA_A$R selectivity-profile than clobazam and better than the canonical benzodiazepine site agonist DZP. It evoked profound antihyperalgesia at doses, which did not cause obvious sedation, muscle relaxation or impairment of motor coordination in mice. Because NDMC is a naturally occurring active metabolite of CBZ in humans, it should be devoid of new serious reactions or toxicity in humans. It therefore appears as a suitable tool compound to assess potential antihyperalgesic efficacy of benzodiazepine site agonists in humans.

Material and Methods

Electrophysiology

The effects of DZP, CBZ and NMDC on currents through recombinant $GABA_AR$s were studied in HEK293 cells (ATCC) transiently expressing rat $GABA_AR$s. HEK293 cells were transfected using lipofectamine LTX 46 (invitrogen). To ensure expression of the γ2 subunit (required for modulation of $GABA_AR$s by BDZs) in all recorded cells, we transfected cells with a plasmid expressing the γ2 subunit plus eGFP from an IRES, and only selected eGFP-positive cells for recordings (see also (Ralvenius et al., 2015). The transfection mixture contained (in µg): 1 αx, 1 βy, 3 γ2/eGFP (used as a marker of successful transfection) or 1 α2, 1 β3, 3 γ2/eGFP. Whole-cell patch-clamp recordings were made 18 – 36 hrs after transfection. Whole-cell patch-clamp recordings of GABA-evoked currents were made at room temperature (20 – 24°C) and at a holding potential of -60 mV. Recording electrodes were filled with solution containing (in mM): 120 CsCl, 10 EGTA, 10 HEPES (pH 7.40), 4 $MgCl_2$, 0.5 GTP and 2 ATP. The external solution contained (in mM): 150 NaCl, 10 KCl, 2.0 CaCl2, 1.0 $MgCl_2$, 10 HEPES (pH 7.4), and 10 glucose. GABA was applied to the recorded cell using a manually controlled pulse (4 - 8 s) of a low sub-saturating GABA concentration (EC_{10}). EC_{10} values of GABA were determined for the four $GABA_AR$ combinations (α1β2γ2, α2β3γ2, α3β3γ2 and α5β2γ2). EC_{50} values and Hill coefficients (n_H) were obtained from fits of normalized concentration–response curves to the equation $I_{GABA} = I_{max}\ [GABA]n_H / ([GABA]n_H + [EC_{50}]n_H)$. I_{max} was determined as the average maximal current elicited by saturating concentration of GABA. DZP, CBZ and NDMC were dissolved in DMSO and subsequently diluted the day of the experiment in external solution and were co-applied with GABA (EC_{10}) without preincubation. Concentration-response curves were fitted to a non-linear regression using the equation for sigmoidal concentration-response curve with variable slope:

$E(C) = E_{max} \cdot [C] \cdot n_H / ([C]n_H + [EC_{50}] \cdot n_H)$, using GraphPad Prism5.

Mice

Experiments were performed in wild-type mice (genetic background C57BL/6J or 129X1/SvJ), and in homozygous quadruple (H→R) $GABA_AR$ point-mutated mice (genetic background 129X1/SvJ). Quadruple point-mutated mice were generated by cross breeding single point-mutated mice described earlier (Rudolph et al., 1999; Low et al., 2000; Crestani et al., 2002).

Animal experiments

All behavioral experiments were performed in 7 - 10 week old female and male mice. Care was taken to ensure equal numbers of female and male mice. All behavioral experiments were made by a male experimenter, blinded either to the genotype of the mice or to their treatment with drug or vehicle. Permission for animal experiments was obtained from the Veterinäramt des Kantons Zürich (license numbers 135/2009 and 126/2012). DZP, CBZ and NDMC were suspended in 0.9% saline, 1% Tween80 and applied orally in all experiments.

Neuropathic pain was evoked by applying a chronic constriction injury (CCI; (Bennett & Xie, 1988)) to the left sciatic nerve proximal to the trifurcation with three loose (5-0 silk) ligatures. Mice, which showed signs of paralysis or which did not develop significant hypersensitivity, were excluded from subsequent experiments. Effects of DZP, CBZ and NDMC on thermal and mechanical hyperalgesia were assessed one week after surgery. Heat hyperalgesia was quantified as the change in the latency of paw withdrawal evoked by exposure of the plantar side of one hindpaw to a defined radiant heat stimulus. Mechanical hyperalgesia was assessed with electronic von Frey filament #7 (IITC, Woodland Hills, CA). Three to four measurements were made for each time point and animal for both heat and mechanical hyperalgesia. Percent maximal possible effect (%MPE) was calculated as follows:

$\%MPE(t) = (E(t)-E_{predrug}))/(E_{preCCI}-E_{predrug})$; E(t), paw withdrawal thresholds or latency at time point t. $E_{predrug}$, E after CCI surgery but before DZP application; E_{preCCI}, E baseline before CCI surgery.

Locomotor activity was measured in an open field arena (10 cm diameter) equipped with four pairs of light beams and photosensors. Drug was applied immediately before placing the animal into the recording chamber. Locomotor activity was analyzed for the time interval between 72 and 120 min after drug administration. Motor control was assessed with a rotarod accelerating from 4 rpm to 40 rpm within 5 min. Sixty min after drug administration mice were placed onto the rotarod. Five measurements were taken per mouse. To assess muscle relaxation, mice were placed with their forepaws onto a metal horizontal wire placed 20 cm above ground. Successes and failures to grab the wire with at least one hindpaw were recorded between 60 and 180 min after drug administration.

NDMC pharmacokinetics

Pharmacokinetic properties of NDMC were evaluated after oral administration of 3, 10 and 30 mg/kg, using the dried blood spot sampling method. This technique allows collecting multiple bleeds from the same animal over a large time window (Déglon et al., 2011). Four µl of whole blood were collected and spotted onto a filter paper card from Whatman (Dassel, Germany) at different time points between 0 and 48 h after NDMC administration. NDMC determination was performed using a fully validated LC-MS-MS method as previously described (Besson et al., 2013). Pharmacokinetic parameters were estimated by a non-compartmental method using WinNonlin® version 5.2 (Pharsight, Mountainview, CA, USA).

Results

$GABA_AR$ subtype selectivity

We first analyzed potency and efficacy of DZP, CBZ and NDMC as positive allosteric modulators of recombinant $GABA_ARs$ expressed in HEK 293 cells (figure 1). All three compounds potentiated currents through α1-, α2-, α3- and α5-$GABA_ARs$. Differences were observed with respect to the potency and efficacy of the three compounds at the four $GABA_AR$ types. DZP potentiated currents through all four $GABA_AR$ subtypes with an EC_{50} between 0.029 and 0.071 µM. CBZ and NDMC were less potent with EC_{50} values between 0.40 and 1.1 µM (CBZ) and between 0.49 and 0.81 µM (NDMC), respectively. Pronounced differences were found between compounds and $GABA_AR$ subtypes with respect to efficacy of potentiation. Potentiation by DZP was highest for α3-$GABA_ARs$ with 237%, while potentiation of the other three $GABA_AR$ subtypes ranged between 100% and 141%.

At sub-saturating DZP concentrations (< EC_{50}, which are probably more relevant to therapeutic effects) the rank order was α1>> α3> α5>> α2. CBZ had very similar efficacies at the four $GABA_AR$ subtypes, but at sub-saturating concentrations potentiation followed a different rank order with α2>> α5> α1>> α3. NDMC had the best α2-/α1-$GABA_AR$ selectivity ratio with respect to efficacy. Efficacy was similarly high for α2 and α3 (253 and 245%) and considerably lower for α1 and α5 (143 and 148%). At sub-saturating concentrations, potentiation was highest for α2, followed by α3 and lowest for α5 and α1. NDMC thus had the most favorable α2-/α1-$GABA_AR$ selectivity profile among the three compounds tested.

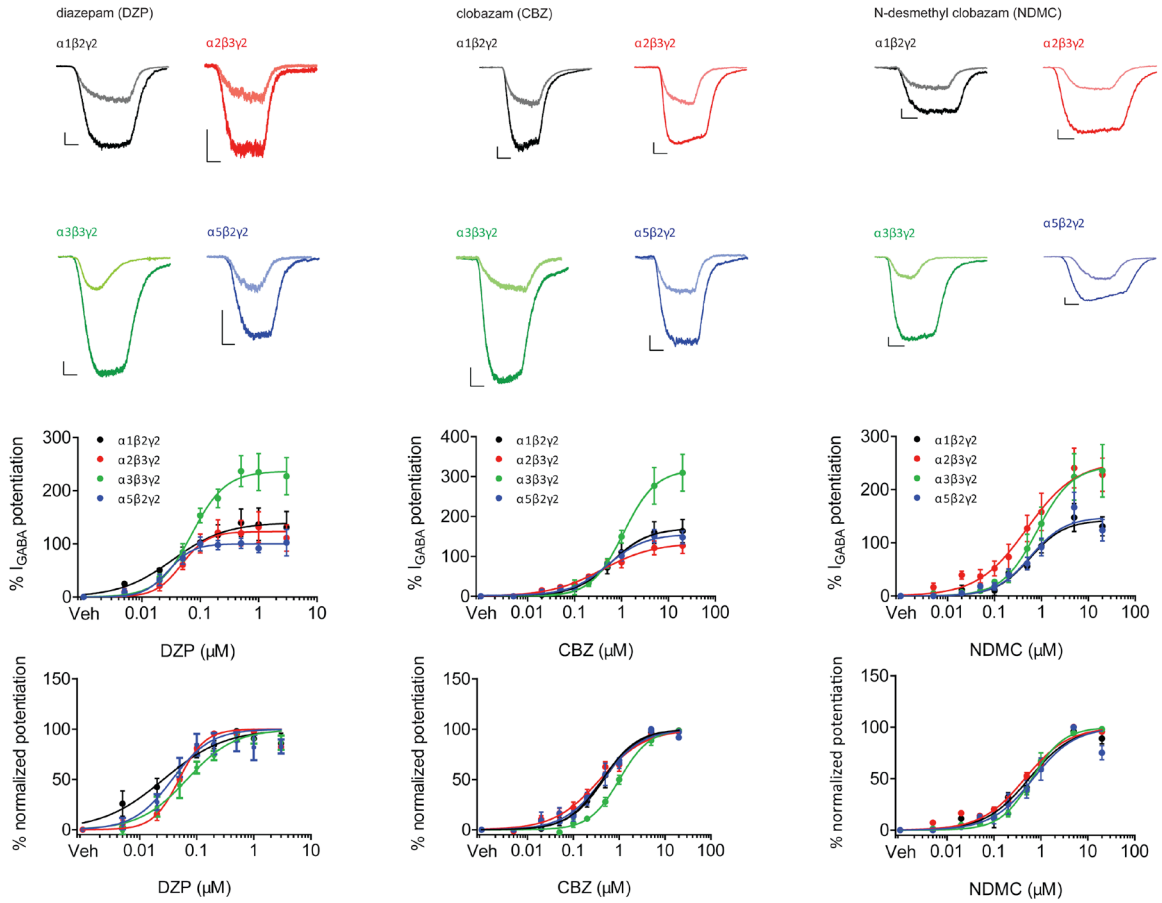

Figure 1. Potentiating effects of DZP, CBZ and NDMC on the four benzodiazepine-sensitive $GABA_AR$ subtypes.
Example traces of currents through recombinant α1/β2/γ2, α2/β3/γ2, α3/β3/γ2, and α5/β2/γ2 $GABA_ARs$ transiently expressed in HEK293 cells before drug application (light tract) and in the presence of DZP (1 μM) (A), CBZ (20 μM) (B) and NDMC (20 μM) (C) (solid trace). GABA concentration was EC_{10} for all experiments. Scale bars, 2s and 200 pA. Concentration response curves of the drugs obtained for all four benzodiazepine-sensitive $GABA_AR$ subtypes. Data are mean ± SEM. Curves represent fits to Hill's equation with the baseline fixed to 0. Numbers of cells tested are between 5 - 7 for all data points.

Sedation

The improved α2/α1 selectivity profile of NDMC should manifest in a reduced propensity to cause sedation. We therefore compared the effects of CBZ and NMDC (3, 10, and 30 mg/kg body weight) on locomotor activity in the open field test (figure 2). We performed these tests in two mouse strains of different genetic background, namely in C57BL/6J mice, which are the standard in most behavioral mouse studies, and in 129X1/SvJ mice, which show higher expression levels of α2-$GABA_ARs$ than C57BL/6 mice (Mulligan et al., 2012). We monitored locomotor activity for 6 hours after drug application.

Statistical analyses were made for the time period between 72 and 120 min because the pain tests described below indicate that this was the time interval of maximal drug effect. In C57BL/6 mice, CBZ induced a dose-dependent reduction in locomotor activity, which reached a statistically highly significant reduction by 70% at a dose of 30 mg/kg ($P < 0.001$) (figure 2 A,B). NDMC did not reduce average locomotor activity at doses up to 10 mg/kg (average changes were < 10%). At 30 mg/kg it caused a moderate reduction by 36% ($P = 0.025$). Reduction in locomotor activity was even less pronounced in 129X1/SvJ mice. In these mice neither, NDMC or its parent compound CBZ caused any measurable reduction in locomotor activity (figure 2 C,D).

Antihyperalgesic actions

Previous studies from our laboratories had demonstrated an analgesic or antihyperalgesic effect of CBZ (Besson et al., 2013). PK/PD analyses had suggested that the antihyperalgesia observed in mice following CBZ administration was at least partially mediated by NDMC, the main metabolite of CBZ. Therefore, we next verified whether NDMC would indeed produce antihyperalgesia. Neuropathic pain was evoked through a chronic constriction injury (CCI) of the left sciatic nerve in wild-type mice. Seven to fourteen days after surgery, four groups of mice were systemically (per os, p.o.) treated with three different doses of NDMC (3, 10, or 30 mg/kg) or with vehicle (figure 3). All mice developed pronounced mechanical and heat hyperalgesia 7 - 14 days after CCI surgery. NDMC dose-dependently reduced both heat and mechanical hyperalgesia (figure 3 A,B). At the maximum dose applied (30 mg/kg), NDMC caused a complete reversal of hyperalgesia.

We next examined whether this antihyperalgesia was indeed mediated by the benzodiazepine binding site of $GABA_A$Rs. This binding site is formed by an interface between one α and the γ2 subunit, and requires the presence of a conserved histidine residue in the $GABA_A$R α subunit. To investigate whether this site was indeed responsible for the antihyperalgesia observed after NDMC, we made use of quadruple point mutated mice in which all four $GABA_A$R α subunits, which can form high affinity benzodiazepine binding site, had been point mutated to disrupt this binding site. In these mice NDMC had completely lost its antihyperalgesic effects (figure 3 C,D).

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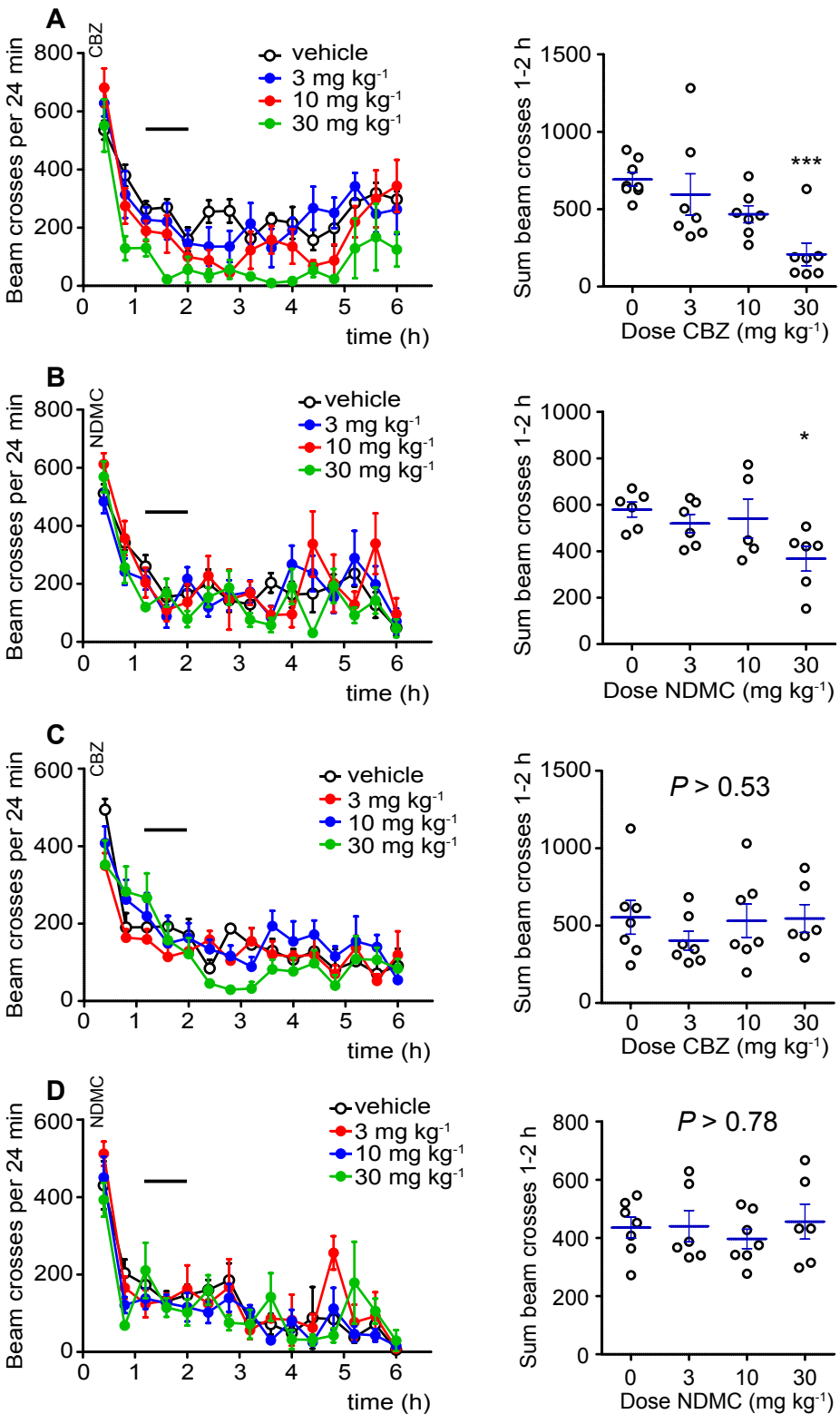

Figure 2. Comparison of sedative effects by CBZ and NDMC in two different mouse strains
Effects of per orally applied CBZ (A) and NDMC (B) in C57BL6/J mice and of CBZ (C) and NDMC (D) in 129X1/SvJ mice on locomotor activity in the open field test. Right side shows statistical analysis. (A) *** $P<0.001$, * $P<0.05$ significant versus vehicle-treated mice (ANOVA followed by Dunnett's post hoc test), $F(3,26)=6.7$ (n=8, 7, 7, 7 for vehicle and 3, 10 and 30 mg/kg CBZ-treated mice) and (B) $F(3,20)=3.2$ (n=6, 5, 6, 6 for vehicle and NDMC-treated mice). All data points are mean ± SEM. (C) $P>0.53$, $F(3,24)=0.58$ (n=7, 7, 7, 6 for vehicle and 3, 10 and 30 mg/kg CBZ-treated mice) and (D) $P>0.78$, $F(3,23)=0.31$ (n=7, 6, 7, 6 for vehicle and NDMC-treated mice). All data points are mean ± SEM.

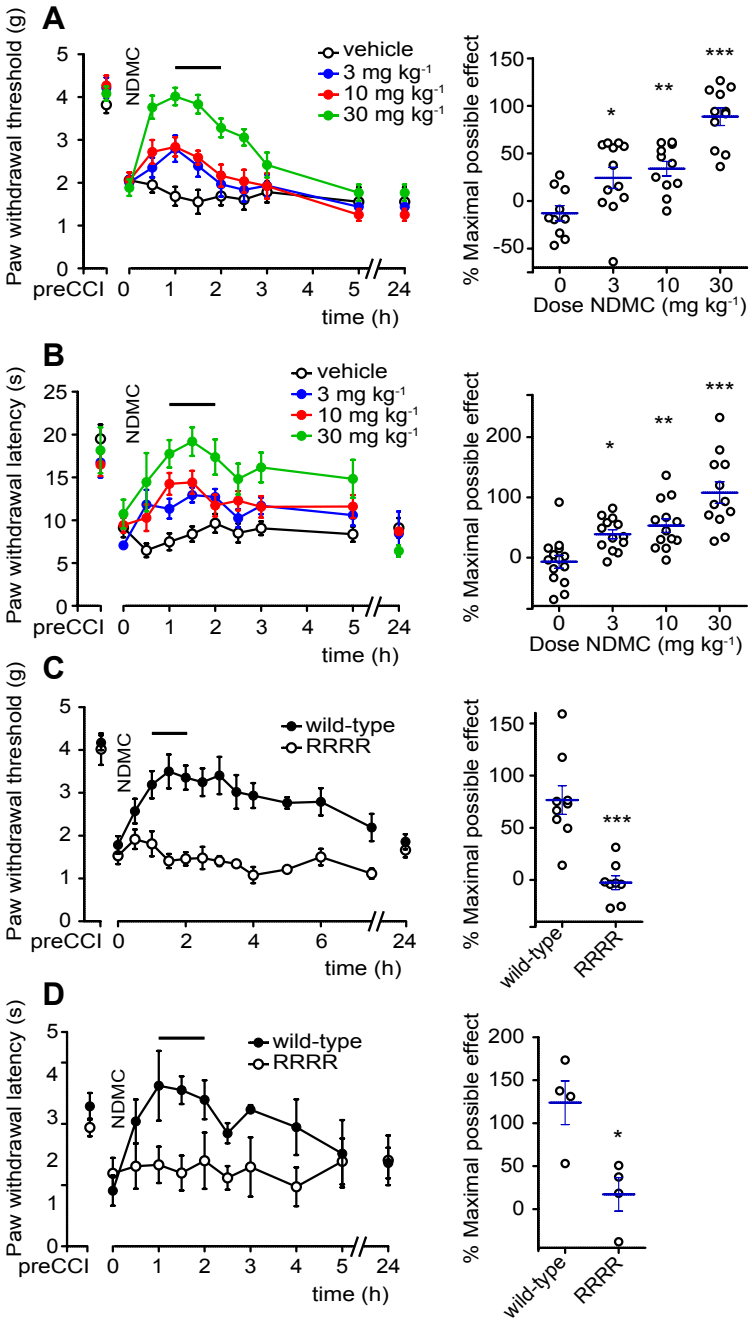

Figure 3. Mechanical and thermal antihyperalgesia by per oral NDMC.
Reversal of hyperalgesia by NDMC assessed with von Frey filaments 7-14 days after CCI surgery. C57BL6/J mice were tested for mechanical hyperalgesia (A), right side shows statistical analysis. *** P<0.001, ** P<0.01 * P<0.05 significant versus vehicle-treated mice (ANOVA followed by Dunnett's post hoc test), F(3,41)=19.8 (n=10, 12, 11, 11 for vehicle and 3, 10 and 30 mg/kg NDMC-treated mice). (B) C57BL6/J mice tested for thermal hyperalgesia 7-14 days after CCI surgery. Right side shows statistical analysis. *** P<0.001, ** P<0.01 * P<0.05 significant versus vehicle-treated mice (ANOVA followed by Dunnett's post hoc test), F(3,49)=15.2 (n=14, 13, 13, 12 for vehicle and 3, 10 and 30 mg/kg NDMC-treated mice). (C) Mechanical antihyperalgesia by NMDC (30 mg/kg) assed in wild-type 129X1/SvJ mice 7-14 days after CCI surgery, right panel shows statistical analysis. *** P<0.001, significant versus vehicle-treated mice (unpaired t-test), n=9 and 8 for wild-type and RRRR mice. (D) Thermal antihyperalgesia by NDMC (30 mg/kg) assed in wild-type 129X1/SvJ mice 7-14 days after CCI surgery, right side shows statistical analysis. * P<0.05, significant versus vehicle-treated mice (unpaired t-test), n=4 and 4 for wild-type and RRRR mice. All data points are mean ± SEM.

NDMC pharmacokinetics

In order to further support that the antihyperalgesia was mediated by NDMC and not by a different down-stream metabolite, we measured plasma levels of NDMC with HPLC following single oral doses of 3, 10 and 30 mg/kg in three mice per group (figure 4). Maximal plasma levels were reached within 2 - 3 hours. Average maximum plasma concentrations were 1.2, 1.9, and 3.3 µg/ml. Plasma half-life was between 4.6 and 5.6 hrs.

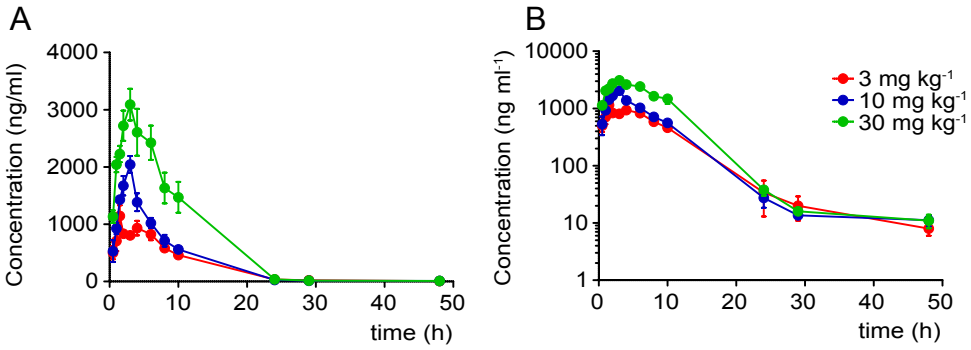

Figure 4. NDMC pharmacokinetics
NDMC whole blood concentrations (mean ± SEM) over time after p.o. administration (A). Display at a logarithmic scale reveals linear elemination kinetics over a large range of concentrations. n=3 per group.

Tab. 1 NDMC PK parameters in whole blood (n=3 mice)

Dose (mg/kg)	Half-life (h)	SD	C_{max} (µg/ml)	SD	AUC (h·µg/ml)	SD	T_{max} (h)	SD
3	5.6	0.6	1.19	2.53	11.15	1.66	2.0	0.9
10	4.6	1.6	1.91	1.34	14.72	4.38	3.0	0.0
30	4.7	0.3	3.31	1.01	32.28	5.09	2.7	0.6

Areas under the curve (AUC) were 11, 15, and 32 h·µg/ml for 3, 10, and 30 mg/kg. Increases in C_{max} and AUC observed with increased doses were thus lower than expected if NDMC followed a linear absorption but elimination followed 1st order kinetics. The t_{max} of about 2.5 hrs and the half-life of around 5 hrs fitted well with the time course of antihyperalgesia following NDMC administration confirming that NDMC did not require further metabolism for its antihyperalgesic action.

Side effect profile

The improved α2/α1 selectivity profile of NDMC suggests that NDMC might exert less or weaker side effects at antihyperalgesic doses than CBZ or DZP. We therefore tested the same three doses of NDMC that had been evaluated for their antihyperalgesic effect for potential impairment of motor coordination and muscle strength in wild-type mice (figure 5). Impairment of motor coordination and muscle relaxation were assessed in the rotarod test and the horizontal wire test, respectively. NDMC showed a dose-dependent trend towards improved performance in the rotarod test. In the horizontal wire test, NDMC showed a trend towards reduced muscle strength, which was not unexpected as facilitation of α2-$GABA_A$Rs causes muscle relaxation. These results indicate that the improved α2/α1 selectivity profile of NDMC obtained *in vitro* indeed translates into reduced side effects *in vivo*.

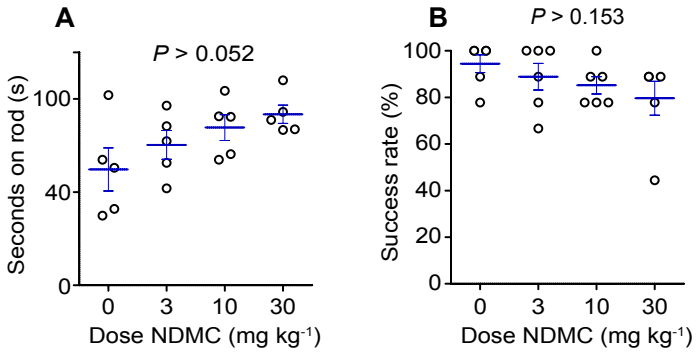

Figure 5. Motor coordination and muscle relaxation.
(A) Effects of NDMC (0, 3, 10 and 30 mg/kg p.o.) on motor coordination (ANOVA followed by Dunnett's post hoc test), F(3,17)=2.5 (n=5, 5, 5, 5 for vehicle and 3, 10 and 30 mg/kg NDMC-treated mice). (B) Effects of NDMC (0, 3, 10 and 30 mg/kg p.o.) on muscle relaxation, (ANOVA followed by Dunnett's post hoc test), F(3,21)=1.4 (n=6, 6, 6, 6 for vehicle and 3, 10 and 30 mg/kg NDMC-treated mice). All data points are mean ± SEM.

Discussion

Diminished synaptic inhibition in the spinal dorsal horn is a common feature of several forms of chronic pain. Restoring synaptic inhibition should therefore constitute a rational strategy for the treatment of many chronic pain syndromes. Fast synaptic inhibition of spinal dorsal horn neurons is mediated by the two amino acids GABA and glycine acting on $GABA_A$Rs and strychnine-sensitive glycine receptors, respectively. While no drugs and very few if any tool compounds specifically modulated inhibitory glycine receptors (Laube et al., 2002; Yévenes & Zeilhofer, 2011), $GABA_A$Rs offer many opportunities for pharmacological

intervention, including positive allosteric modulators among them the benzodiazepines. Previous work from our groups and from several other laboratories has demonstrated that a facilitation of fast GABAergic synaptic transmission in the spinal cord effectively reverses pathologically heightened pain sensitivity in rodents (reviewed in Zeilhofer et al., 2012). Similar antihyperalgesia is however not observed after systemic administration of benzodiazepines in humans (Corrigan et al., 2012; Richards et al., 2012). Our recent study employing triple $GABA_AR$ point mutated mice provides strong evidence that the lack of a clinically relevant antihyperalgesic effect of systemically administered benzodiazepines originates from dose limiting sedative effects of classical benzodiazepines (Ralvenius et al., 2015). In these triple point mutated mice, in which only either α1- or α2-$GABA_AR$ subtype remained benzodiazepine-sensitive, has demonstrated that half maximal sedation (via α1-$GABA_ARs$) occurs already at doses 20 times lower that those required for half maximal antihyperalgesia (mediated by via α2-$GABA_ARs$). This finding is consistent with human studies demonstrating that benzodiazepine-induced sedation occurs in humans already at very low levels of receptor occupancy (Pauli et al., 1991). This study has shown that clinically relevant antihyperalgesia in human requires the development of benzodiazepine site ligands with high efficacy at α2-$GABA_ARs$ and negligible activity at α1-$GABA_ARs$. This concept is consistent with two recent studies that showed that classical benzodiazepines (CBZ and clonazepam) exert some albeit weak analgesic or antihyperalgesic effects in humans at weakly sedating doses (Besson et al., 2015; Vuilleumier et al. 2013). Unequivocal evidence for a pain relieving effect in humans will however depend on proof-of-concept studies with drugs having a significantly improved α2/α1 selectivity. At present no such compounds have been approved for use in humans.

The present study suggests that NDMC might be suitable compound for human proof-of-concept trials. NMDC exhibited stronger efficacy at α2-$GABA_ARs$ than CBZ and DZP. This result is largely consistent with previous reports on the affinity and efficacy of CBZ and NDMC at $GABA_AR$ subtypes expressed in Xenopus oocytes (Jensen et al., 2014; Hammer et al., 2015). In mice, NDMC evoked pronounced antihyperalgesia at doses, which did not induce significant reductions in locomotor activity. Because NDMC is a naturally occurring metabolite of the clinically used benzodiazepine CBZ (Caccia et al., 1979), occurrence of new side-effects unknown for CBZ is highly unlikely. At present, it is however unknown whether the weak effects NDMC in the open field test predict also low sedative effects in humans. CBZ and NDMC exhibit very similar potency and efficacy at sedative α1-

$GABA_AR$s. Furthermore, the efficacy of both compounds at α1-$GABA_AR$s is very similar to that of DZP, a highly sedating benzodiazepine. Nevertheless, at doses causing significant analgesia, NDMC reduced locomotor activity less than its parent compound CBZ (this study) and also less than DZP (Knabl et al., 2009). Our previous study in triple point mutated mice indicates that locomotor activity in the open field test is not only affected by α1-$GABA_AR$s but also by α2-$GABA_AR$s. While α1-$GABA_AR$s reduce activity, activation of α2-$GABA_AR$s increases activity possibly through their anxiolytic effects (see also chapter 1 figure 3). It is thus likely that the smaller impact on locomotor activity of high doses of NDMC results from increased activity at α2-$GABA_AR$s rather than from reduced activity at α1-$GABA_AR$s. This interpretation is consistent with results obtained in 129X1/SvJ mice. In these mice expression of α2-$GABA_AR$s is about twice as high as in C57BL/6J mice that were used for the open field tests shown in figure 2 (data not shown). These findings may suggest that NDMC might not be less sedating than CBZ or DZP. Assessing the three compounds in triple point mutated mice with only α1-$GABA_AR$s left benzodiazepine-sensitive should help answering this question at least for mice.

On the positive side, it should be noted that NDMC potentiated currents through α2-$GABA_AR$s already at much lower concentrations than currents through the other three $GABA_AR$ subtypes tested. It is thus possible that NDMC exerts α2-$GABA_AR$-mediated antihyperalgesia already occur at doses that are devoid of significant sedative effects. Whether the shift in potency of NDMC at α2-$GABA_AR$s that was observed *in vitro* translates into clinically significant analgesia at non-sedative doses in humans, remains to be confirmed.

In summary, our study shows that NDMC exhibits an improved α2/α1-$GABA_AR$ selectivity profile *in vitro*. Our behavioral experiments suggest that this improved molecular profile translates to the *in vivo* situation. Whether the improvement is large enough to warrant a proof-of-concept study in human volunteers or patients remains to be determined.

Author contributions

WTR performed all behavior tests, except initial antihyperalgesia tests, which were done by MB and AM. MAA did the electrophysiological experiments. MB and AM did the NDMC analytics and analyzed the data. MAA performed and analyzed the electrophysiology experiments. HUZ designed experiments, analyzed data and wrote the manuscript. All authors made comments on the manuscript.

Acknowledgements

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GENERAL DISCUSSION

Benzodiazepines have been used for decades to treat humans suffering from a variety of brain disorders including anxiety, insomnia and epilepsy. Attempts to develop benzodiazepines with increased selectivity for non-α1GABA$_A$ receptors have initially been made to reduce the side effects, i.e. to develop anxioselective, non-sedating benzodiazepines. Recently, several reports suggest that such novel subtype-selective benzodiazepines might also be used for the treatment of diseases which do not benefit from classical benzodiazepines, such as schizophrenia, depression and stroke (Rudolph and Knoflach, 2011). One such potential new indication is also chronic pain. The data presented in this thesis support the idea that novel benzodiazepines with selective activity at the α2-GABA$_A$ receptors (or in addition at α3-GABA$_A$ receptors) should be well-suited for the treatment of chronic pain. This concept is well in-line with previous preclinical studies of our laboratory and from others (Knabl et al., 2008; Munro et al., 2008; Knabl et al., 2009; Di Lio et al., 2011; Nickolls et al., 2011; Hofmann et al., 2012; Besson et al., 2013; Paul et al., 2014; de Lucas et al., 2015).

All these studies clearly indicate that benzodiazepine site ligands with a high intrinsic activity at α2-GABA$_A$ receptors and low activity at α1-GABA$_A$ receptors reduce inflammatory and neuropathic hyperalgesia in mice and rats. The great majority of these studies, including most of the experiments performed in our laboratory, used withdrawal responses to quantify antihyperalgesia. Although changes in these responses correlate in general well with pain perception, they do not necessarily allow the conclusion that the drugs under study do also reduce pain sensation. Furthermore, these test measure threshold responses only, and may be of limited value for the assessment of ongoing and suprathreshold pain. Nevertheless, several experiments done in our laboratory indicate that activation of α2-GABA$_A$ receptors does not only interfere with spinally coordinated withdrawal responses but also with supraspinal pain processing. Results very similar to those obtained with withdrawal response were seen in the formalin test, which exposes the animal to suprthreshold pain and triggers supraspinally coordinated behaviors such as biting or licking responses. Furthermore, one of the first "α1-sparing" benzodiazepine site agonists, L-838,417 (McKernan et al., 2000), reduced not only

withdrawal responses in inflamed rats but also reduced pain-correlated brain activity in BOLD fMRI experiments (Knabl et al., 2008). To further substantiate our hypothesis that activation of α2-$GABA_A$ receptors reduces not only spinally-mediated pain responses but also conscious pain experience, conditioned place preference experiments (King et al., 2009) might be employed in the future.

Throughout most of the thesis, I refer to the pain suppressing effect of subtype selective benzodiazepines as "antihyperalgesia". This is meant to emphasize the point that these compounds do not suppress acute nociceptive pain (i.e. in our experiments they have no effect on pain thresholds in the contralateral non-inflamed or non-injured paw). This term also acknowledges the fact that what is measured in our experiments is mostly a change in heightened sensitivity to noxious stimuli, usually called hyperalgesia. The use of the term "antihyperalgesia" should however not imply that these compounds would not interfere with on-going pain in inflammatory or neuropathic condition. The conditioned place preference experiments mentioned above will help to address also this point.

Neuropathy and inflammation can cause diminished inhibition in the spinal dorsal horn through a variety of mechanisms (Zeilhofer et al., 2009). In all cases when diminished inhibition and pain occur through reduce responsiveness of neurotransmitter receptors, reduced synthesis or release of GABA, it is conceivable that drugs facilitating the activity of $GABA_A$ receptors would alleviate pain. However, when diminished inhibition results from disrupted chloride homeostasis, the case is more complicated. A previous report has suggested that the increase in intracellular chloride concentration following peripheral nerve damage can turn the effect of $GABA_A$ receptor activation from inhibition to excitation (Coull et al., 2003). In this case, facilitation of $GABA_A$ receptors would increase rather than alleviate pain. It should be pointed out that in none of our experiments we did observe any evidence for a pronociceptive effect of benzodiazepine site ligands. This lack of a pain enhancing effect is also consistent with a recent report from Allan Basbaum's laboratory, which shows that transplantation of GABAergic precursor cells into the spinal cord of neuropathic mice reverses pathological pain (Bráz et al., 2012). These results do not challenge the concept of a disturbance of chloride homeostasis in neuropathic pain. They rather suggest that the inhibitory effect of GABAergic input is weakened but does not reverse into excitation. Nevertheless, a combination with positive allosteric modulators of KCC2 which promote restoring physiological chloride homeostasis may increase the therapeutic efficacy of GABAergic drugs in neuropathic pain patients (Price and Prescott, 2015).

A question central to the translational aspect of this thesis is whether facilitation of $GABA_A$ receptors does also have an impact on pain in humans. Classical non-selective benzodiazepines do not exert clinically relevant analgesia in patients (Corrigan et al., 2012; Richards et al., 2012). Our data obtained in the triple $GABA_A$ receptor point mutated mice suggest dose-limiting sedation as the major reason for the absence of clinically relevant analgesic properties of classical benzodiazepines in human patients. However, species differences cannot be fully excluded *a priori*. Evidence supporting the translatability comes from several different directions. Previous studies from our laboratory have demonstrated that α2-$GABA_A$ receptor-mediated antihyperalgesia originates largely, if not exclusively, from a spinal site of action (Paul et al., 2014). A morphological study by (Waldvogel et al., 2010) analyzed the expression pattern of the $GABA_A$ receptor subunits in the human CNS. The expression of all α subunits, of β2/3 and of the γ2 subunit reported in this study is remarkably similar to what we and others have previously reported for mice and rats (Bohlhalter et al., 1996; Paul et al., 2012). Other supporting evidence comes from a human genetics study. Smith et al. (Smith et al., 2012) tested the association of more than 350 genes with fibromyalgia in 496 patients and 348 controls. Two SNPs in the *GABRB3* gene, which encodes for the $GABA_A$ receptor β3 subunit, showed the strongest and seventh strongest association among a total of 2760 SNPs tested. Because the β3 subunit is contained in most α2-$GABA_A$ receptors, this finding supports a critical role of α2-$GABA_A$ receptors also in human pain processing. Finally, direct evidence supporting a pain relieving effect of benzodiazepine site ligands also in humans comes from recent studies by (Vuilleumier et al., 2013; Besson et al., 2015), who tested the effects of two rather weakly sedating benzodiazepines, clobazam and clonazepam, on experimental pain in human volunteers. Both studies report weak but statistically significant analgesic or antihyperalgesic effects at mildly sedating doses.

Given the great promises of preclinical studies it is surprising that none of the non- or less sedative benzodiazepine site agonists developed by pharmaceutical industry got approved for human therapy. The three most promising compounds in development probably were MRK-409, TPA023 and TPA023B, all developed by Merck Sharp & Dohme to treat anxiety (Atack, 2010). Unfortunately, in humans MRK-409 was sedative at relatively low levels of receptor occupancy due to remaining agonistic activity the benzodiazepine binding site of α1$GABA_A$ receptors. TPA023 lacks α1 efficacy, but the human trial with this compounds was terminated due to unexpected toxicity issues. At least one of the compounds, TPA023B

showed no signs of sedation in humans (Atack et al., 2011), and was last reported on in 2011 to be in Phase I clinical trials against anxiety disorders and schizophrenia (Rudolph and Knoflach, 2011). Concert Pharmaceuticals has been developing a drug called CTP-354, which is a deuterated version of L-838,417, for the treatment of spasticity (concert pharmaceuticals, 2015). The company was expecting to launch two separate Phase II trials last year, one evaluating the drug for treatment of spasticity caused by spinal cord injury and the other spasticity caused by multiple sclerosis.

Although TPA023B might be at least a useful compound to test antihyperalgesic efficacy in human pain patients, publicly available safety data are not sufficient to obtain permission for clinical studies. One of the aims of this thesis was therefore to explore alternative strategies based on the assumption that benzodiazepine metabolites occurring in humans after administration of a classical benzodiazepine might exert a better $GABA_A$ receptor subtype selectivity profile. A pharmacodynamic/pharmacokinetic study on clobazam in mice revealed that the antihyperalgesic effects seen after clobazam administration were not caused by clobazam itself but by an active metabolite (Besson et al., 2013). The major metabolite of clobazam in man is N-desmethyl clobazam (Caccia et al., 1979) As analyzed in detail in this thesis, N-desmethyl clobazam does not only reduces antihyperalgesia in mice but also exhibits a more favorable selectivity ratio at α2 versus α1 $GABA_A$ receptors. Because N-desmethyl clobazam is a naturally occurring metabolite of clobazam, occurrence of new side-effects unknown from clobazam is highly unlikely. It may thus be considered a suitable compound proof-of-concept studies in humans.

Alternative strategies to further promote the translation of the concept of GABAergic analgesia to patients may involve the testing of suitable compounds in non-rodent animal species. In particular dogs often suffer from chronic pain conditions, mainly caused by degenerative joint diseases. TPA023B, has already been given to dogs in the context of pharmacokinetic studies and was well tolerated (Atack et al., 2011). It should therefore be possible to evaluate this compound in veterinary patients.

In summary, the use of the triple $GABA_A$ receptor point-mutated mice has allowed to predict *in vivo* actions of yet to be developed subtype-selective agents with unprecedented precision. The identification of N-desmethyl clobazam as a potentially suitable compound for clinical proof-of-concept studies may foster the translation of these discoveries to clinical therapy.

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Society of Neuroscience Annual Meeting 2013 (San Diego, USA)

Antihyperalgesic Effects of Systemic Diazepam Assessed in Complex $GABA_A$ Receptor Mutant Mice

NeuPSIG 4th International Congress on Neuropathic Pain 2013 (Toronto, Canada)

Antihyperalgesic Actions of Diazepam in Mice Carrying Multiple Diazepam-Insensitive $GABA_A$ Receptor Types

FENS Forum of European Neuroscience 2012 (Barcelona, Spain)

Neutralizing Nerve Growth Factor to treat chronic inflammatory pain

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Awards

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Original Research Publications

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Review articles

Zeilhofer HU, **Ralvenius WT**, Acuña MA (2015) Restoring the spinal pain gate: $GABA_A$ receptors as targets for novel analgesics. *Adv Pharmacol* 73, 71-96

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